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HIV-1 Env and HLA-C interaction is crucial in modulating viral infectivity

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Summary

È noto che alcuni polimorfismi del sistema HLA giocano un ruolo cruciale nell'eziopatogenesi e nella prognosi di numerose malattie infettive, fra le quali l'AIDS (Sindrome da Immunodeficienza Acquisita).

Recenti studi hanno evidenziato una forte correlazione fra i livelli di espressione di HLA-C e il controllo della replicazione del virus dell'immunodeficienza umana (HIV-1). Alti livelli di espressione sono stati correlati con un miglior controllo dell'infezione, mentre bassi livelli sono stati associati con una progressione più rapida della malattia. Inoltre, è noto che la molecola HLA-C, presente sull'*envelope* di HIV-1, in associazione con la glicoproteina Env, è in grado di aumentarne l'infettività. Il ruolo protettivo di alti livelli di espressione di HLA-C sembra essere in contraddizione con il ruolo dell' HLA-C stesso nell'aumentare l'infettività virale quando incorporato nel virione. Ciò potrebbe essere dovuto alla presenza di diverse conformazioni dell' HLA-C. È infatti noto che diverse varianti alleliche dell'HLA-C presentano una diversa stabilità di legame con la β_2 microglobulina (β_2m) e il peptide. In particolare, l'HLA-C può presentarsi associato alla β_2m e al peptide, costituendo un complesso che svolge un ruolo chiave nell'attivazione del sistema immunitario, oppure come free chain, dissociato dal complesso. I primi risultati di questo lavoro hanno dimostrato che la proteina Env di HIV-1 è in grado di associarsi all'HLA-C quando presente nella conformazione di free chain. L'ipotesi testata nello studio prevede l'esistenza di un'associazione fra la suscettibilità all'infezione da HIV-1 e le diverse varianti alleliche di HLA-C che possono essere preferibilmente presenti o come complesso trimerico o come free chain. Individui con varianti di HLA-C aventi una forte stabilità come trimero completo mostrerebbero una maggiore immunità contro HIV-1 e una ridotta infettività virale, mentre soggetti con varianti dell'HLA-C che facilmente si dissociano dalla β_2m e dal peptide mostrerebbero una ridotta risposta immunitaria nei confronti di HIV-1 e la produzione di virioni maggiormente infettivi. Nel suo complesso, questo studio fornisce nuove informazioni che potrebbero rivelarsi utili per la progettazione di nuove strategie vaccinali e approcci terapeutici contro HIV-1.

Abstract

Introduction

HLA-C plays a crucial role in the progression of HIV-1 infection. Host genetic HLA-C variants, appear to be associated with a different ability to control HIV-1 infection. A higher HLA-C expression is associated with a better activation of cytotoxic T lymphocytes (CTLs) and of Killer Immunoglobulin like receptors (KIR) on NK-cells, which lead to a better HIV-1 infection control. *Vice-versa*, a lower HLA-C expression leads to a rapid progression toward AIDS. In addition, different HLA-C alleles present different binding stabilities to $\beta_2$microglobulin (β_2m)/peptide. Noteworthy, some HLA-C highly expressed/protective alleles are also stably bound to β_2m/peptide, while some low expressed/non-protective variants present an unstable bond to β_2m/peptide. Finally virions lacking HLA-C have reduced infectivity and increased susceptibility to neutralizing antibodies.

Experiments

In the present work, it was first characterized the association between HLA-C and HIV-1 Env. We investigated if HIV-1 infection involves HLA-C free chains or the heterotrimeric complex, and to this purpose the A3.01 cell line and its HIV-1-infected counterpart ACH-2, as well as PM1 cells, were used as *in vitro* infection model. HEK-293T β_2m negative cells, generated using CRISPR/Cas9 system, were used to produce HIV-1 pseudoviruses and to test their infectivity. Then, the proportion between HLA-C associated to β_2m and HLA-C presents as free chains on the cell surface was characterized on PBMC from healthy donors, bearing both Stable or Unstable HLA-C alleles. In addition, PBMC were tested for their ability to support HIV-1 infection *in vitro*.

Results

HIV-1 infection induces the appearance of HLA-C free chains on the surface of infected cells, which may be responsible for the increased HIV-1 infectivity. HIV-1 Env-pseudotyped viruses produced in the absence of β_2m, thus lacking HLA-C on their envelope, were less infectious than those produced in the presence of β_2m.

By analysing PBMC from healthy donors, differences in HLA-C heterotrimers stability and HLA-C expression levels were found. Finally, it was reported that R5 HIV-1 virions produced by PBMC having Unstable HLA-C alleles were more infectious than those produced by PBMC having the Stable variants.

Conclusions

The outcome of HIV-1 infection might depend both on the HLA-C surface expression levels and on HLA-C/β_2m/peptide binding stability.

According to this model, PBMC carrying low expressed/Unstable HLA-C alleles have a high proportion of HLA-C free chains on their surface that raises viral infectivity and, at the same time, a low proportion of HLA-C heterotrimeric complexes which leads to a poor control of HIV-1 infection, and thus to a rapid progression toward AIDS.

Contents

HIV-1 Introduction

Infection disease and its determinants

During an infection many aspects are important to determine the disease severity, duration and the final outcome. The intrinsic pathogenic power of the microorganism, as well as its infecting load, plays a key role. Furthermore, the ability of the host to counteract the infection is crucial. This is dependent on the status of his host immune system. Many genetic polymorphisms are involved in different infectious diseases (Chapman and Hill 2012). Many of these have a key role both in the replicative cycle of the organism, and in the host immune response. Some individuals, based on their genetic variants, are able to better control a specific infection, than others. Thus, different subjects show different outcomes of the same infection.

Specifically, the Major Histocompatibility Class I molecules (MHC-I) influence several chronic inflammatory and autoimmune conditions, such as type I diabetes, multiple sclerosis and Crohn's disease. In addition, MHC variants confer susceptibility to many infectious diseases, including malaria and AIDS (Traherne 2008).

Mechanisms used by viruses to escape antigen presentation

The host immune responses evolved to fight viral infections, and in parallel viruses co-evolved in order to escape the reaction of the immune system. During this ongoing evolution, viruses developed several mechanisms to entry and to replicate in the infected host cells, despite the host immune system activation (Lucas, Karrer et al. 2001). Different viral proteins can directly affect the expression of MHC-I on the infected cells, hampering the host immune response. Furthermore, viral proteins can mediate the internalization or degradation of MHC-I molecules, making the infected cells resistant to cytolysis mediated by Cytotoxic T Lymphocytes (CTLs). Infected cells are still susceptible to lysis

mediated by Natural Killer (NK) cells. Another strategy implemented by viruses, to evade the host immune system, is to hamper the antigenic peptides expression on the cell surface, impairing the activation of the immune system. Viruses can block proteolysis processes or viral antigens transport, by interfering with the activity of the proteasome or of other peptidases, involved in the generation of antigenic peptides, leading to the generation of epitopes unable to bind the carrier Transporter associated with Antigen Processing (TAP). Thus, their translocation into the Endoplasmic Reticulum (ER) is blocked and the antigenic peptide can not associate to MHC-I molecules and be presented on the membrane of infected cell (Verweij 2015). An additional mechanism exploited by different viruses to affect the antigenic peptides generation process, is the down-regulation of TAP itself, which leads to the reduction of the formation of MHC-I/peptide complexes that activate the CTL response (Hansen and Bouvier 2009) (Figure 1).

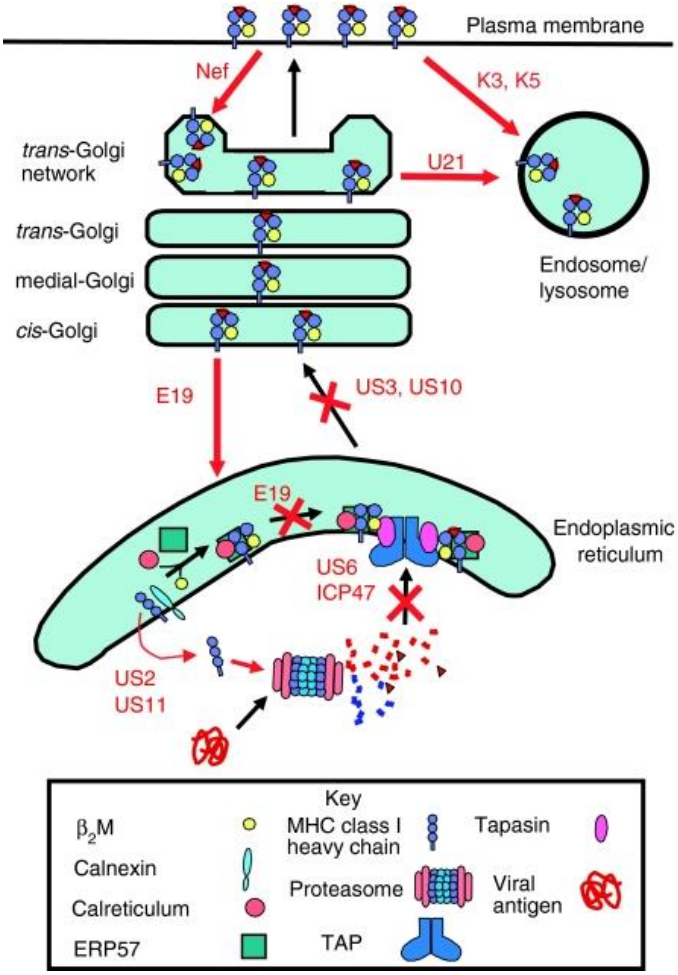


Figure 1. Viral inhibition of the MHC class I antigen presentation pathway. Viruses have developed proteins that inhibit the MHC class I pathway at different steps. US2 and US11 (Human cytomegalovirus, HCMV, proteins) translocate the MHC class I heavy chain into the cytosol where it is degraded by the proteasome. ICP47 (herpes simplex virus, HSV, protein) and US6 (Human cytomegalovirus, HCMV, protein) inhibit peptide translocation by TAP. E19 (Adenovirus E3 protein) inhibits MHC class I association with TAP, E19 also inhibits MHC class I trafficking by retrieving MHC class I molecules from the cis-Golgi. Similarly, US3 and US10 (Human cytomegalovirus, HCMV, proteins) inhibit the ER export of class I molecules. U21 (human herpes virus 7 protein) drives MHC class I molecules to the lysosome. Nef (HIV protein) down-regulates MHC class I surface expression. K3 and K5 (Kaposi's sarcoma-associated herpesvirus, KSHV, proteins) down-regulate MHC class I molecules leading to their degradation (Hewitt 2003).

The Acquired Immune Deficiency Syndrome

The Acquired Immune Deficiency Syndrome (AIDS) is a pathological condition caused by the Human Immunodeficiency Virus (HIV) infection. AIDS is characterized by a progressive impairment of the host immune system, as a consequence of the continue depletion of circulating $CD4^+$ T-cells. Because of the declining immune response, several opportunistic infections and/or cancers affect the patients, leading to death (Gallo and Montagnier 2003).

HIV origin and classification

AIDS had a zoonotic origin, since HIV evolved from Simian Immunodeficiency Virus (SIV) from Chimpanzee. HIV has been classified into two different species, named HIV-1 and HIV-2. The origin of these viruses was investigated through molecular phylogenetic studies, revealing that HIV-1 evolved from a strain of Simian Immunodeficiency Virus (SIVcpz) within a particular subspecies of the Chimpanzee (*Pan troglodytes*) (Gao, Bailes et al. 1999), while HIV-2 originated from SIVsm, which infects Sooty mangabeys (*Cercocebus atys*) (Hirsch, Edmondson et al. 1989). The SIVcpz appears to be the co-infection result of the same animal with lentivirus of the red capped mangabey (SIVrcm) and a virus of the greater spot-nosed monkey (SIVgsn) lineage (Heeney, Dalgleish et al. 2006). HIV-1 sequence analyses dated the HIV origin most probably in 1908 (Worobey, Gemmel et al. 2008) (Figure 2).

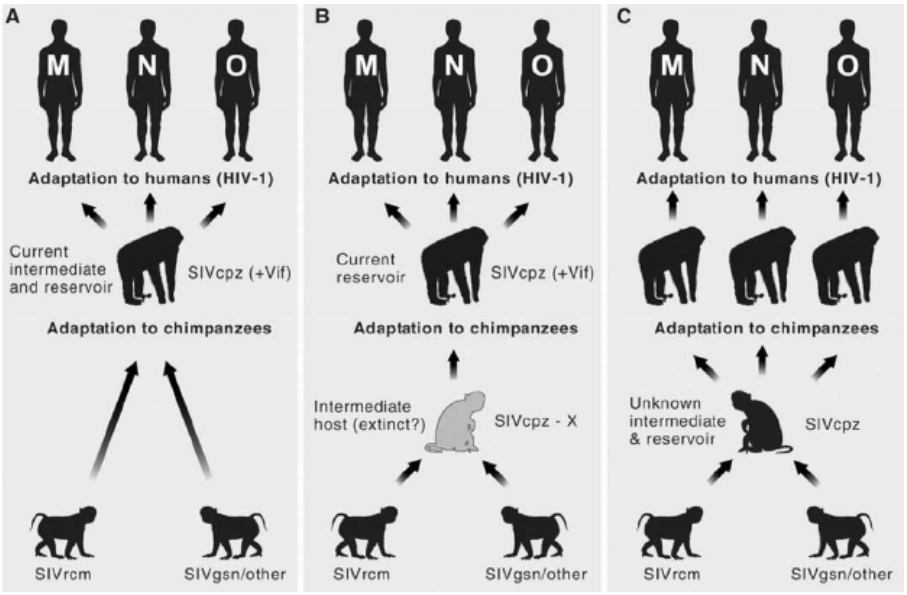


Figure 2. Image represents three possible alternative ways of cross-species transmissions giving rise to SIVcpz. A) Recombination requires co-infection of a single *Pan troglodyte* with one or more SIVs. B) The SIVcpz recombinant develops and is maintained in an unknown primate host. C) Transfer through an intermediate host (yet to be identified) that is the actual reservoir of introduction of SIVcpz into communities of *P. troglodytes* (Heeney, Dalgleish et al. 2006).

Retrospectively traces of HIV-1 came from a 1959 blood sample and a 1960 tissue sample collected in the Democratic Republic of Congo (Zhu, Korber et al. 1998, Kalish, Robbins et al. 2004, Worobey, Gemmel et al. 2008). Subsequent to HIV-1 discovery, it was classified in four different groups: M (main) responsible of most AIDS cases in the world, O (outlier), N (non-M, non-O) and P defined as rare group (Plantier, Djemai et al. 2009). Moreover, the M group has evolved into different clades or subtypes (A to K) (Osmanov, Pattou et al. 2002). Recent subtypes present different genomic segments, derived from recombination of more than one subtype (Smith, Richman et al. 2005). Different circulating recombinant forms (CRFs) and unique recombinant forms (URFs) do exist, due to the co-infection with different HIV-1 isolates in some patients (Tebit and Arts 2011).

HIV-1 is highly heterogeneous within infected individuals due to high viral load, rapid turnover rate and an error-prone reverse transcriptase enzyme which lacks proofreading activity. High variability is also due to recombination, that moves mutations between viral genomes which can lead to major antigenic shifts or changes in virulence (Tebit and Arts 2011).

HIV-1 discovery

At the beginning of the 80s, in California and New York a weird increase of rare diseases and malignancies was reported in young, homosexual men (1981). All of them, presented a dramatically decrease of circulating $CD4^+$ T-cells, responsible of the immunological deficit. They were also affected by *Pneumocystis carinii* infection, Kaposi's sarcoma and persistent lymphadenopathy (Fauci, Schnittman et al. 1991). The researchers defined this condition 'Acquired Immunodeficiency Syndrome' (AIDS) and they found its etiology in a retrovirus (Gallo 2002, Montagnier 2002).

In that period, two retroviruses, HTLV-I and HTLV-II, characterized by preferential tropism for T-cells, were known. In the laboratory National Cancer Institute in Bethesda, directed by Robert Gallo, blood samples from AIDS affected patients resulted negative for cross-reactivity with HTLV proteins (Broder and Gallo 1984). Consequently, the new type of virus was called HTLV-III, to be distinguished from the other two types (Beilke 2012). Meanwhile, at the Pasteur Institute in Paris, Luc Montagnier and coworkers, identified in cell cultures, derived from a patient with serious immunodeficiency and lymphadenopathy, a new virus they named Lymphadenopathy-Associated Virus (LAV) (Barre-Sinoussi, Chermann et al. 1983). In the same year Gallo and his coworkers finalized the isolation and culture of viral isolates in $CD4^+$ T-cells. These research groups were the first that provided a detailed description of the newly discovered retrovirus, identified as the etiologic agent of AIDS. In 1986, due to the discovery of another retrovirus, called HIV-2, the form previously isolated was called HIV-1 (Clavel, Guyader et al. 1986). The identification of the etiological agents of AIDS, led to rapid advances toward the full characterization of HIV-1. Soon after, the first anti-HIV-1 agent, azidothymidine (AZT), was used to treat AIDS patients (Mitsuya and Broder 1989). Francoise Barré-Sinoussi and Luc Montagnier, were awarded, in 2008, with the Nobel Prize in Medicine and Physiology for HIV-1 discovery (Abbadessa, Accolla et al. 2009).

HIV-1 epidemiology

Nowadays, HIV-1 continues to represent a global health problem. From its discovery, it has caused an estimated 39 million deaths worldwide spreading by sexual, parenteral, and perinatal routes. As reported by the World Health Organization (WHO) about 37.0 million people were living with HIV in 2016, of which about 2 million were children (Figure 3).

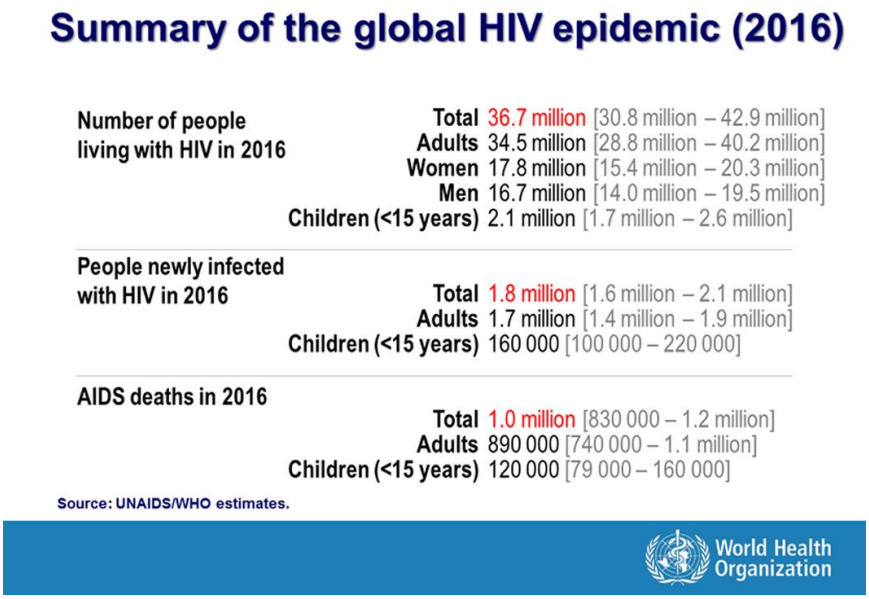


Figure 3. Table shows the people living with HIV, people newly infected with HIV-1 and AIDS death in 2016. (Source: http://www.who.int/hiv/data/en/).

HIV-1 structure

The HIV-1 virion is a spherical retrovirus with a diameter of ~100–150 nm. Each viral particle is composed by an external envelope associated to the matrix surrounding the capsid, which encloses the viral genome and other enzymes (Frankel and Young 1998) (Figure 4).

The envelope is a phospholipid bilayer derived from the infected cell membrane. The intermediate layer, anchored to the inner side of the envelope, is called matrix and it is formed by the viral protein p17. The inner layer, named capsid, is a conical shape core, and it is made by the p24 viral protein assembled in about 250

hexameric subunits. It contains the viral genome, bound to the nucleocapsid protein (p7) and other viral proteins necessary for viral replication, such as the Reverse Transcriptase (RT), the Protease (PR) and the Integrase (IN).

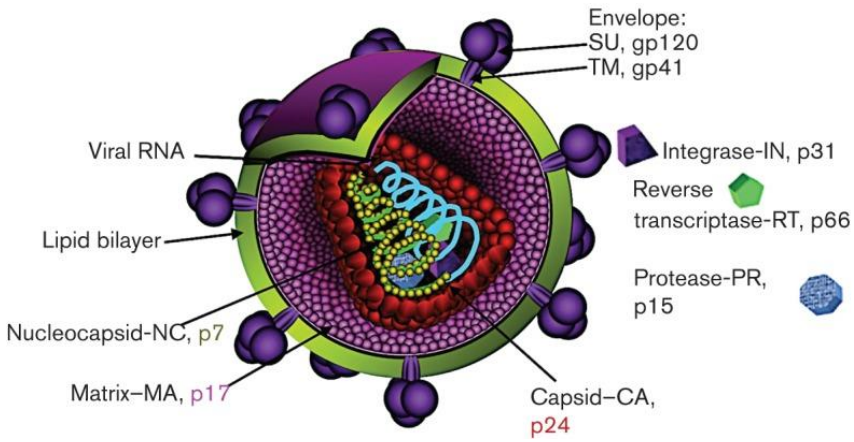


Figure 4. HIV-1 virion structure.
Schematic representation of the mature HIV virion. The representation is not to scale (Steckbeck, Kuhlmann et al. 2013).

HIV-1 genome

The HIV-1 genome, is about 9 Kb long. Its structure follows the typical pattern of the *Retroviridae* family: it contains three genes called Gag, Pol and Env from 5' to 3' end. These sequences are flanked by two Long Terminal Repeat (LTR) regions (Frankel and Young 1998). The Gag and Env genes encode for proteins necessary to the structure of the virions, while the Pol gene codes for the viral enzymes (Frankel and Young 1998). Furthermore, the HIV-1 genome encodes for other six proteins: Rev, Tat, Vif, Vpr, Vpu, Nef (Malim and Emerman 2008). Specifically, Rev and Tat are regulatory proteins, while Vif, Vpr, Vpu and Nef are involved in the viral infection *in vivo* (Figure 5).

Below are reported in detail the transcript products and their functions.

- Gag (group specific antigen) is a polyprotein (p55), which is processed to form different proteins: the matrix (p17 or MA), the capsid (p24 or CA), the nucleocapsid (p7 or NC) and the p6 (Ganser-Pornillos, Yeager et al. 2008). MA is located in the N-terminal component of the Gag polyprotein and it is important to target Gag and Gag-Pol precursor polyproteins to the

plasma membrane before viral assembly. The second component of the Gag polyprotein is CA, and it forms the core of the viral particles. NC is the third component of the Gag polyprotein and coats the genomic viral RNA inside the virion core. p6 encompasses the C-terminal 51 amino acids of Gag and it is crucial for Vpr incorporation during viral assembly (Frankel and Young 1998).

- Pol (polymerase) is synthesized as a unique Gag-Pol protein of 160 KDa, which is then processed to obtain three different proteins: the Protease, the Reverse Transcriptase and the Integrase (Frankel and Young 1998). PR cleaves at many sites to produce the final MA, CA, NC, and p6 proteins from Gag and PR, RT, and IN proteins from Pol. The RT catalyzes both DNA-dependent and RNA-dependent DNA polymerization reactions and has an RNase H domain which cleaves the RNA portion of RNA-DNA hybrids generated during the reaction. IN, after reverse transcription, catalyzes a series of reactions to integrate the viral genome into the host chromosome (Frankel and Young 1998).
- Env (envelope) is produced as the gp160 precursor, then processed to obtain gp120 and gp41 (Pantophlet and Burton 2006). The gp120 protein, exposed on the viral surface, is important for virus attachment to the host cell; while gp41 is a trans-membrane protein that plays a key role in entry (Chojnacki, Staudt et al. 2012). The gp120 and the gp41 glycoproteins form a trimeric complex by non-covalent interaction (Frankel and Young 1998).
- Rev (regulator of the expression of the virion) is important for the expression of other structural proteins. It recognizes the Rev Responsive Elements (RRE) and binds the unspliced viral mRNAs coding for structural proteins, helping the mRNA export from the nucleus (Strebel 2003). In the absence of Rev, the transcripts are spliced, leading to the production of small regulatory proteins.
- Tat (trans-activator of transcription) is a regulatory protein. It binds to an RNA hairpin, known as TAR (trans-activating response element), located at the 5’ end of the nascent viral transcripts and enhances the processivity

of transcribing polymerases. Tat increases production of HIV mRNAs ~100-fold and thus is essential for viral replication (Frankel and Young 1998). Tat controls the elongation, the transport in the cytoplasm and the translation of the viral RNA (Strebel 2003, Nilson and Price 2011).

- Vif (viral infectivity factor) is a protein important for the viral replication. Vif promotes the ubiquitination and degradation of the host restriction factor APOBEC3G, avoiding the introduction of mutations into the viral genome (Madani and Kabat 1998) (Malim and Emerman 2008). Therefore, Vif deficient viruses are able to infect the target cell, but they are not able to replicate (Strebel 2003).
- Vpr (viral protein r) is a small basic protein (14 kDa). Despite its small size, it has been shown to have different functions in the course of the viral infection (Guenzel, Herate et al. 2014) by interfering with the anti-retroviral defences of the host cells (Zhao, Kang et al. 2014).

  Vpr facilitates HIV-1 replication in infected cells (Goh, Rogel et al. 1998), induces the blockade of the cell cycle at G2 phase and affects the accuracy of the reverse-transcription process (Poon and Chen 2003). It has been proposed a Vpr role in the transport of the viral pre-integration complex (PIC) to the nucleus (Le Rouzic and Benichou 2005) but additional evidences suggest that Vpr does not play a crucial role in the nuclear transport of HIV-1 PIC. Indeed, it has been reported that HIV-1 bearing either precise mutations or deletion of entire Vpr has only reduced but not entirely abolished its replication in primary macrophages (Jayappa, Ao et al. 2012).
- Vpu (viral protein u) mediates the degradation of CD4 molecules which would trap the Env protein in the Endoplasmic Reticulum (ER) (Bour and Strebel 2003). Vpu is important for the virus “budding” process, since mutations in Vpu are related with persistence of viral particles at the host cell surface. Membrane molecules such as tetherin (CD317) can bind Vpu-deficient HIV-1 virions and prevent viral release (Malim and Emerman 2008). Recently, has been reported that Vpu downregulates the

HLA-C surface expression upon HIV-1 infection (Apps, Del Prete et al. 2016).

- Nef (negative factor) is an N-terminally myristoylated protein, which promotes the endocytosis and the lysosomal degradation of CD4 (Frankel and Young 1998, Geyer, Fackler et al. 2001) and down-regulates the expression of MHC-I molecules, preventing the lysis of infected cells by the cytotoxic T-lymphocytes (Doms and Trono 2000, Malim and Emerman 2008). Nef enhances viral infectivity and replication in PBMC (Feinberg), alters the T-cell activation state and the macrophage signal transduction pathways (Swingler, Mann et al. 1999), inhibits the immunoglobulin class switching (Qiao, He et al. 2006) and associates with several components of the endocytic pathways (Chaudhuri, Lindwasser et al. 2007). Two independent studies reported that Nef is able to promote HIV-1 infection by preventing SERINC3 and SERINC5 incorporation into virions (Rosa, Chande et al. 2015, Usami, Wu et al. 2015).

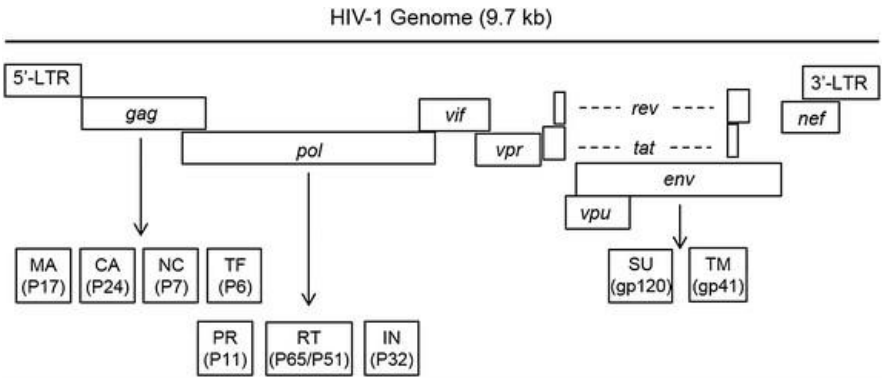


Figure 5. HIV-1 genome structure.
Arrows indicate cleaved protein products. Dashed lines represent RNA splicing (Nkeze, Li et al. 2015).

HIV-1 infection cycle

The HIV-1 infection cycle, which occurs after the recognition of the receptor and co-receptors on the cell surface, leads to the generation of new viral particles from the infected cell. The entire HIV-1 cycle could be summarized in three consecutive stages (Figure 6).

1. Entry

The HIV-1 entry in the host cell is mediated by the interaction between the HIV-1 gp120 glycoprotein and the CD4 receptor expressed on the host cells (Dalgleish, Beverley et al. 1984). CD4 is expressed on different cells, such as T-lymphocytes, macrophages, Dendritic Cell (DC), monocytes and microglial cells. After the interaction between gp120 and CD4 a structural change in the gp120 occurred, leading to the exposition of the co-receptor binding site (Sierra, Kupfer et al. 2005). The principal co-receptors, exploited by HIV-1, are the seven trans-membrane CC chemokine Receptor type 5 (CCR5) (Deng, Liu et al. 1996) and the CXC chemokine Receptor type 4 (CXCR4) (Feng, Broder et al. 1996). The recognition of one of these co-receptors induces the insertion of the gp41 N-terminal hydrophobic fusion-peptide region into the target cell membrane, leading to the fusion and entry of the viral particle (Doms and Trono 2000). The usage of these two distinct co-receptors, defines the HIV-1 tropism. HIV-1 isolates which mainly replicate in primary T-cells (T-tropic) use the CXCR4 co-receptor, while HIV-1 isolates which mainly infect macrophages (M-tropic) exploit the CCR5 co-receptor (Berger, Murphy et al. 1999). In the natural course of HIV-1 infection, R5-tropic variant appear first, while X4-tropic variants emerge later, when virus is already adapted to the host (Mariani, Vicenzi et al. 2011). New infections are generally established by HIV-1 variants that use CD4 and CCR5 as a co-receptor (Schuitemaker, Koot et al. 1992). Even when both R5 and X4 tropic variants are present in an infected subject, generally only the R5-tropic variants are transmitted to the recipient (Zhu, Mo et al. 1993, van't Wout, Kootstra et al. 1994). X4-tropic variants, which emerge later, are associated

with an accelerated $CD4^+$ T-cells decline and a more rapid disease progression (Bozzette, McCutchan et al. 1993, Koot, Keet et al. 1993).

Furthermore, HIV-1 isolates that already expose the conserved region required for the co-receptor binding, do exist. These variants, do not require CD4 receptor expression to infect the target cells, and for this reason, they are called CD4-independent. Due to the exposure of the hidden conserved region, necessary to the binding with the co-receptors, these variants are more susceptible to neutralization. For this reason, it could be possible to suppose that CD4 recognition represents a mechanism evolved subsequently, to escape the host immune system (Iyengar and Schwartz 2012). The HIV-1 Env high variability, and the virus ability to conceal the conserved regions, represent the major obstacle for the vaccine development against HIV-1.

2. Post entry

After the HIV-1 fusion event the Reverse Transcriptase enzyme catalyses the reverse transcription of the genomic viral ssRNA into double stranded DNA (provirus).

Since RT is a key enzyme for viral replication, it was identified as a promising target molecule for drug treatment.

Due to the lack of proofreading activity, RT introduces in each new synthetized DNA molecule, an average of five mutations, determining the production of novel variants and contributing to the evolution of virus (Smyth, Gargon et al. 2012). These mutations can confer an advantage or a disadvantage to the virus, leading to the formation of replication-incompetent viral species, as well as drug resistant viral variants, which represent an important obstacle during the long-term therapy (Domingo, Estrada et al. 2012). In addition, viral replication is quick and an average of 10^9 new viral particles are produced every day. Thus, in every infected subject, several closely-related virus variants do exist, that could confer drug and immune resistance.

After retro transcription, the proviral dsDNA is incorporated into the Pre-Integration Complex (PIC) with other HIV-1 proteins and transported to the nucleus where it is integrated into the host genome (Schroder, Shinn et al. 2002).

Once integrated, the virus can follow two different fates depending on the availability of host cell transcription factors (Williams and Greene 2007). The virus can replicate or establish a latent infection. When the virus infects a resting cell, as T-lymphocytes developing in memory cells, a latent infection occurs, generating an important long-lived reservoir for HIV-1. These immunological sanctuaries represent an important obstacle for complete viral eradication (Dahl, Josefsson et al. 2010). *Vice-versa* when the virus infects a cell in active proliferation it can replicate.

3. Replication and maturation

After integration, the proviral DNA is transcribed by the host RNA polymerase, strongly enhanced by Tat, starting from the promoter sequences, located in the LTR regions (Frankel and Young 1998).

At the beginning of the infection, small regulatory proteins, such as Tat, Rev and Nef, are translated after a complete splicing of the viral RNA. Instead, during the late phases of the HIV-1 replication cycle, viral genes are transcribed and viral RNAs are exported from the nucleus to the cytoplasm, where single spliced proteins are synthesized (Env, Vif, Vpr, and Vpu). At the same time, unspliced variants such as Gag and Gag-Pol are produced (Purcell and Martin 1993). Both Gag and Gag-Pol polyproteins mediate the viral assembly at the plasma membrane (Ganser-Pornillos, Yeager et al. 2008). Furthermore, Gag promotes Gag-Gag interaction, the encapsidation of two copies of the viral RNA genome per virion and the association with Env (Freed 1998).

Nef and Vpu play a key role in the Env recruitment into the nascent virions (Freed 1998). Immediately after the budding Gag and Pol proteins are obtained by the cleavage of viral protease permitting to the virion to became completely infectious. During this essential maturation process a structural reorganization of the viral proteins occurs, converting the capsid morphology from a spherical shape to a conical core (Sakuragi 2011).

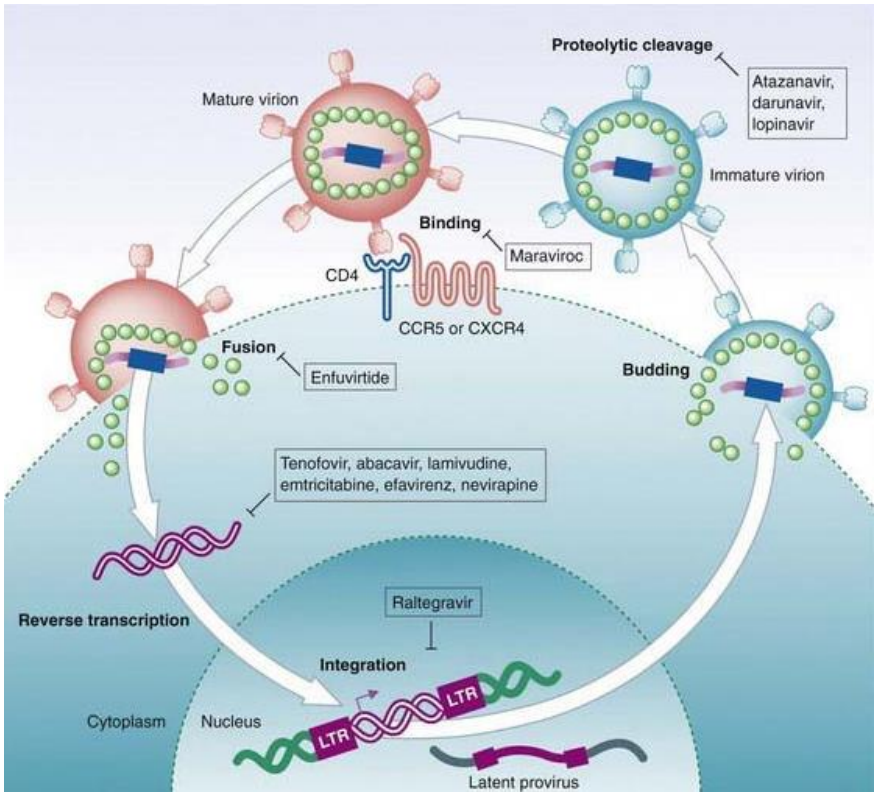


Figure 6. HIV replication cycle.
Infectious steps: binding, fusion, reverse transcription, integration, transcription, translation, assembly, budding, release and maturation. In addition, ART targeting mechanisms are indicated (Volberding and Deeks 2010).

HIV-1 transmission

HIV-1 could be transmitted either through vertical and horizontal transmission. HIV-1 can be directly transmitted from one infected person to another through body fluids containing HIV-1 virions and HIV-1 infected cells, such as, semen, vaginal secretions, blood or breast milk. Thus, the main ways of HIV-1 horizontal transmission are represented by sexual contacts or the sharing of needles to inject drugs with an HIV-1-infected person. Furthermore, the risk of HIV-1 transmission correlates to viremia level: it is as high as the risk of transmission, hence an undetectable viral RNA is most likely associated with a low risk of transmission (Brenner, Roger et al. 2007). Fortunately, new therapies can control the viral load and keep it at low, undetectable levels reducing the risk of transmission and limiting the HIV-1 spread.

Vertical transmission may occur from an HIV-1 infected mother to the newborn, especially when the mother is not receiving the therapy before, at birth, or during breastfeeding (Gouws and Cuchi 2012).

Natural course of HIV-1 infection

The natural course of HIV-1 infection is characterized by three phases: the acute phase, the chronic phase, and the AIDS (Figure 7).

- The initial acute phase (the firsts weeks after infection) is characterized by viral replication, that leads to high levels of viremia and a low number of circulating $CD4^+$ T-cells, which are infected and killed by the virus (Guadalupe, Reay et al. 2003). During this phase HIV-1 can spread undisturbed from the mucosal barriers to lymph nodes, generating permanent reservoirs (Chun, Engel et al. 1998). Furthermore, the virus spreads directly from one infected cells to an uninfected one through the formation of virological synapses (Jolly, Kashefi et al. 2004, Sattentau 2008).

  The acute phase is not characterized by specific symptoms and it can be easily confused with a common flu. Consequently, newly infected subjects remain unaware of their highly viremic status, representing a risk for HIV-1 transmission to other people (Brenner, Roger et al. 2007).
- After the reaction of the immune system the viral load reaches a stable level, called 'viral load set point', the chronic phase starts. This phase lasts differently in different patients (Mellors, Rinaldo et al. 1996). Generally, a higher viral set point is associated with a fast progression towards AIDS, *vice-versa* a lower viral set point leads to a slow progression toward AIDS. It has been reported that a subject affected by HIV-1 may carry about 30000 RNA copies/ml of blood (Mellors, Rinaldo et al. 1996).
- The AIDS phase, the symptomatic one, is characterized by a strong increase of the viral load as well as by a corresponding decrease of $CD4^+$ T-cells (below 200 cells/ml). This phase is characterized by a deep

immunodeficiency, resulting in a higher susceptibility to opportunistic infections, mainly caused by Human Herpes Virus 8 (HHV-8), Cytomegalovirus (CMV), Human Papilloma Virus (HPV), *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Candida albicans*. HHV-8 is responsible of a Kaposi's Sarcoma, CMV generally causes retinitis, an eye infection that can lead to blindness, while HPV is associated with a higher risk to develop cervical or anal cancer. *Pneumocystis carinii* pneumonia (PCP) is a life-threatening lung infection that usually affect people with weakened immune system. More than three-quarters of people affected by AIDS will develop PCP if they do not receive treatment to prevent it. In addition, the parasite is able to infect the ears, eyes, skin, liver and other organs (Source: Office of Communications National Institute of Allergy and Infectious Diseases National Institutes of Health Bethesda, Maryland 20892).

Moreover, a biologic synergy between HIV and tuberculosis (TB) does exist. HIV-induced immunosuppression increases susceptibility to active TB infection, and at the same time active TB infection increases HIV progression and risk of death. *Mycobacterium tuberculosis* most often causes a chronic pneumonia, characterized by cavities similar to abscesses. This microorganism can affect other organs, establishing latent infection. Reactivation in these organs can lead to local disease (e.g., in the lymph nodes, meninges, bone, pericardium, peritoneum or intestine, and urogenital tract) (Source: https://aidsetc.org/guide/mycobacterium-tuberculosis).

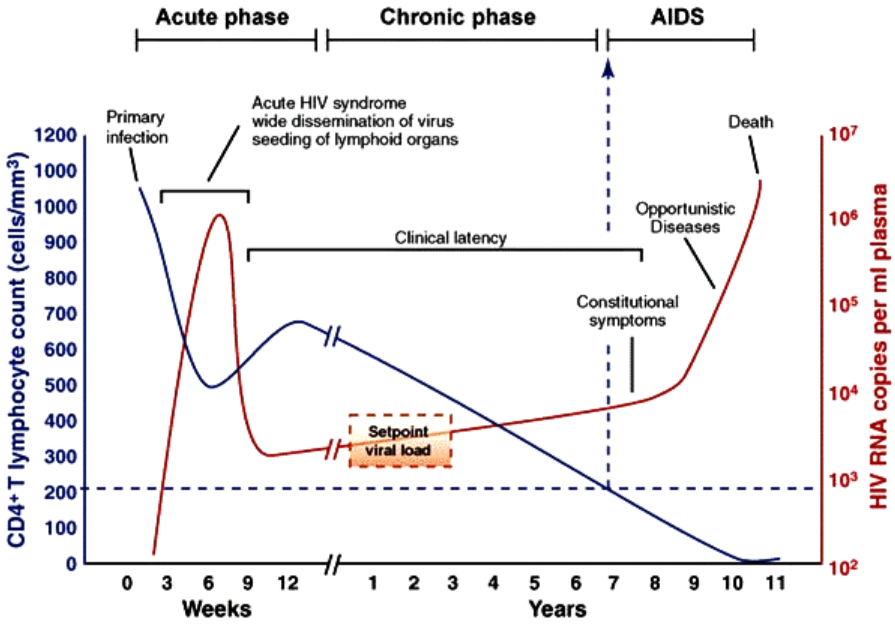


Figure 7. Natural course of HIV-1 infection.
Scheme of the phases of the natural course of HIV-1 infection: acute phase, chronic phase and AIDS phase. Viral load and $CD4^+$ cells count are reported in red and blue respectively (O'Brien and Hendrickson 2013).

Host response against HIV-1

The human immune system is divided into two categories: innate and adaptive. The innate immune responses are the first non-specific line of defense against invading pathogens. Adaptive immunity refers to antigen-specific immune response, which occurs after the innate response.

1. Innate immunity

The first defence that the host uses to counteract the HIV-1 infection is mediated by the innate immunity, a non-specific system that recognizes particular non-self antigens (called PAMPs, Pathogen-associated molecular patterns) shared by exogenous organisms, such as carbohydrate, proteins or lipid structures.

During an HIV-1 infection the most important PAMP is the RNA viral genome that activates the Toll-Like Receptor (TLR) 7 and 8. Activation of these receptors leads to a stimulation of plasmacytoid Dendritic Cell (pDC) and consequently to the release of type 1 interferons (IFN-α/IFN-β) and other pro-inflammatory cytokines able to trigger the immune response (Chang and Altfeld 2010).

Different polymorphisms in TLRs associated with different HIV-1 disease outcomes do exist (Oh, Jessen et al. 2008).

Another system employed by the host during the first line defence against HIV-1 infection is represented by restriction factors. These are anti-viral proteins encoded by the host cell that counteract or 'restrict' viral replication. Some of them are APOBEC3G, TRIM5α and SAMHD1. APOBEC3G is a cytidine deaminase and it is responsible for C-to-U hypermutation in viral genomes (Browne, Allers et al. 2009), TRIM5α is able to recognize the HIV-1-CA leading to the multimerization of TRIM5α, premature uncoating of the virion core, and activation of TRIM5 E3 ubiquitin ligase activity (Luban 2012). SAMDH1 is a deoxynucleotide-triphosphate (dNTP) hydrolase, which restricts HIV-1 RT reducing levels of dNTPs (Mlcochova, Sutherland et al. 2017). At the same time, another borderline, component of innate immunity involved in the early control of HIV-1 is represented by the Natural Killer cells (NK). NK cells have attributes of both innate and adaptive immunity. They have germline-encoded receptors like a component of innate immunity and rearranged receptors as adaptive immunity (Vivier, Raulet et al. 2011).

NK cells were originally described as cytolytic effector lymphocytes, which can directly induce the death of virus-infected or tumour cells in the absence of specific immunization. NK cells activation is mediated by the lack of MHC-I molecules, exposed on the cell surfaces, a situation that can occur when cells are infected or are becoming tumoral (Vivier, Tomasello et al. 2008).

HIV-1 developed a sophisticated escape mechanism to avoid the killing of infected cells both from the NK cells and from the CTLs. HIV-1 Nef down-regulates the dominant T-cell receptor ligands HLA-A and B but maintains a spare amount of HLA-C, which can bind the inhibitory Killer Immunoglobulin like receptors (KIR) on the NK cells (Cohen, Gandhi et al. 1999, Schaefer, Wonderlich et al. 2008, Carrington and Alter 2012).

2. Adaptive immunity

The activation of the innate immunity leads to the activation of the adaptive immunity, which acts through two different mechanisms: humoral and cellular.

The most important humoral response to HIV-1 infection, is against the viral gp120 and gp41 glycoproteins. Since conserved epitopes are hidden, antibodies against gp120 and gp41 target a region with high variability and thus they are often unable to control the infection. In addition, glycosylation and highly variable loops in conserved epitopes (Wei, Decker et al. 2003, Pantophlet and Burton 2006) make these antibodies ineffective and lead to the selection of HIV-1 escape mutants. On the contrary, antibodies against the Env conserved regions, like the receptor and co-receptor binding sites (e.g. b12 and 2G12 antibodies) and the gp41 membrane proximal external region (e.g. 2F5 and 4E10 antibodies) are rarely found in HIV-1 positive patients. These antibodies usually appear in the late stage of AIDS. They have a wide potent neutralizing activity and are able to block multiple HIV-1 strains (Shattock and Moore 2003). Strangely, controllers patients have low levels of these antibodies (Pereyra, Addo et al. 2008, Lambotte, Ferrari et al. 2009). Thus, it seems that humoral response could play only a marginal role in the control of HIV-1 infection (Sierra, Kupfer et al. 2005). However, HIV-1 specific mucosal IgA have been found in many exposed uninfected individuals, correlating with possible immune protection (Devito, Broliden et al. 2000, Miyazawa, Lopalco et al. 2009). In general, anti HIV-1 antibodies can act through different mechanisms such as complement mediated lysis, phagocytosis or Antibody Dependent Cellular Cytotoxicity (ADCC).

Differently from the humoral response, the cell-mediated immunity plays an important role in HIV-1 infection control. $CD8^+$ T-cells (Cytotoxic T Lymphocytes, CTLs) are an important cell type, involved in eliminating HIV-1 infected cells. They are activated through their T Cell Receptor (TCR) by viral peptides presented by MHC-I molecules at the cell surface. They can directly induce the lysis of the infected cells by the release of perforin and proteases. CTLs can also act indirectly through the production of cytokines, chemokines or other anti-HIV-1 factors that inhibit viral replication (Levy 2011). The importance of CTLs in controlling HIV-1 infection is demonstrated by the observation that

HIV-1 infected monkeys, artificially lacking CTLs, present a rapid increase of viremia (Schmitz, Kuroda et al. 1999). The CTL response is highly specific, thus HIV-1 variants carrying mutation in the viral proteins within CTL epitopes are quickly selected during the acute phase of the infection (Price, Goulder et al. 1997). It has been proposed that the quality of the CTL response, rather than the quantity, is important in the control of viremia (Bangham 2009). Many studies demonstrated that individuals who control HIV-1 infection possess T-cells able to trigger a multifunctional response, not only through a cytolitic mechanism, but also through the production of cytokines and chemokines (Walker and McMichael 2012).

In addition, HIV-1-specific $CD4^+$ T-cell response is triggered during the acute infection, but it appears to be reduced or lost in most patients, during the course of the infection. This effect could be due to dysregulated activation, proliferation failure, HIV-1 induced T-cells anergy, antigen-induced cell death, direct infection or apoptosis (Cantin, Fortin et al. 1997, Esser, Bess et al. 2001). For instance, HIV-1 Tat down-regulates MHC-II expression and therefore may lead to viral induced anergy by impairing antigen recognition (Kanazawa, Okamoto et al. 2000). However, an inverse correlation between HIV-1 specific $CD4^+$ T-cells response and plasma viral load has been described, suggesting that $CD4^+$ mediated immune response has a protective role (Connick, Marr et al. 1996).

Cell host membrane proteins incorporation in HIV-1 virion

HIV-1 incorporates different host proteins while budding out from infected cells (Tremblay, Fortin et al. 1998). Cellular proteins found in purified HIV-1, HIV-2 and SIV virions include β_2m, HLA-DR, MHC-I and actin (Arthur, Bess et al. 1992). Details of HIV-1 specific incorporation of host cellular proteins are not well established. Some proteins are simply passively incorporated into the nascent virion due to their contiguity to budding sites (Tremblay, Fortin et al. 1998, Ott 2008), while other proteins are selectively incorporated by direct association with viral proteins. A specific incorporation happens, for example, for the adhesion

molecule ICAM-1 (Beauséjour and Tremblay 2004) or other cytoskeleton-anchored proteins (Ott, Coren et al. 1996, Tremblay, Fortin et al. 1998).
The selective acquisition of host proteins by HIV-1 strongly contributes to viral pathogenesis. Other surface proteins like MHC-I and MHC-II (Cantin, Fortin et al. 1997, Esser, Bess et al. 2001), CD80, CD86 (Giguere and Tremblay 2004), Galectin (Ouellet, Mercier et al. 2005) are incorporated into the nascent HIV-1 virion, to promote its infectivity by favouring the virus attachment to target cells.
It has been shown that MHC-I and MHC-II stimulating T-cells trough the TCR without the appropriate second co-stimulatory signal might result in cell anergy and apoptosis. This effect could explain the $CD8^+$ and $CD4^+$ T-cells impairment observed in HIV-1 infected patients (Esser, Bess et al. 2001, Cantin, Methot et al. 2005).

Host genotype and HIV-1 susceptibility

Different individuals show different susceptibilities to HIV-1 infection. Genetic and viral factors, as well as immune responses, are associated with differences in disease progression. The host genetic variation explains about 20% of interpersonal susceptibility to HIV-1 infection (Fellay, Ge et al. 2009). As previously mentioned, the viral set point is related to the progression towards AIDS symptomatic phase (Fellay, Ge et al. 2009). A higher viral load is followed by a rapid progression of the disease, *vice-versa* a lower viral load is associated with a slow progression towards AIDS (Lyles, Munoz et al. 2000, Langford, Ananworanich et al. 2007). According to this feature four different classes of subjects can be defined:

- Rapid Progressors (RP), infected individuals that develop AIDS within few years from HIV infection (generally presenting more than 100000 copies of HIV-RNA/ml of blood);
- Long Term Non Progressors (LTNP), infected individuals that are able to control the virus for many years (generally having <5000 HIV-RNA copies/ml);

- Elite Controllers (EC), infected individuals characterized by undetectable levels of viremia (<50 copies/ml in the absence of therapy);
- Exposed uninfected (EU), not infected individuals although they were exposed to the virus.

Among the host genetic variants which affect the HIV-1 infection outcome, a deletion in the CCR5 gene (named CCR5Δ32) is the most important. In the mid-1990s, it was found that the CCR5Δ32 allele leads 'to nearly complete resistance to HIV-1 infection' (Stephens, Reich et al. 1998). This genetic mutation shows geographical traits: it is apparently absent among East Asians, Africans and Amerindians, while it is found in up to 14%, in specific northern populations of Eurasia (Novembre, Galvani et al. 2005). It was speculated that this CCR5Δ32 variant could have conferred resistance to *Yersinia pestis*, explaining its prevalence in North Eurasia populations. Since the mechanism of Yersinia-induced macrophage apoptosis involved CCR5 receptor, the CCR5Δ32 mutation would be an attractive candidate for a strong selective pressure 600-700 years ago. Other possibilities are *Salmonella*, *Shigella*, *Mycobacterium tuberculosis*, and Smallpox virus which similarly target macrophages (Stephens, Reich et al. 1998).

The CCR5Δ32 variant confers resistance against HIV-1 since the truncated protein is not expressed on the cell membrane preventing the viral entry into the host cell (Martinson, Crain et al. 2003). This mutation, due to the redundancy of the chemotactic system, does not compromise the physiological functionality of the immune system.

Moreover, other important genetic variants related to HIV-1 infection control are located in the MHC-I genes (Carrington and O'Brien 2003, Fellay, Ge et al. 2009, Leslie, Matthews et al. 2010, Pereyra, Jia et al. 2010). It has been demonstrated that a greater selective pressure is set to the virus from the HLA-B locus, characterized by the highest number of polymorphisms. For instance, the HLA-B*35 allele is related with a rapid progression towards AIDS; on the contrary, the presence of HLA-B*27 or B*57 alleles is associated with a slow progression to the disease (Carrington and O'Brien 2003). A cohort study conducted in Sub Saharan Africa reported that the association of B*57:03 with a lower viral load occurs in concomitance with HLA-C*18, but it is completely lost

in its absence. Furthermore, HLA-B*58:01 allele too has been associated with a lower viral load in the presence of C*03, rather than C*07 (Lazaryan, Lobashevsky et al. 2006, Zipeto and Beretta 2012). These findings suggest that the additive effect of different HLA alleles plays a key role in AIDS progression (Leslie, Matthews et al. 2010).

HIV-1 treatment

Nowadays, HIV-1 infected people have an almost normal life expectancy thanks to the availability of a wide variety of drugs (Figure 8). The most important classes of drugs for HIV-1 treatment are reported below (Orsega).

Nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs): they are nucleoside or nucleotide analogues, able to block the nascent retro transcribed DNA filament.

Protease inhibitors (PIs): their mechanism role is based on interference with the protease activity necessary for the viral maturation.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs): they bind non-competitively to the RT enzyme, blocking its catalytic site.

Entry inhibitors: they interfere with the binding of HIV-1 to its receptor/co-receptor or prevent its fusion with the host cell membrane.

Integrase inhibitors: they act blocking the integration of HIV-1 DNA into the host cell genome.

The most important advance in the field of HIV-1 therapy is represented by the introduction of the Highly Active Anti-Retroviral Therapy (HAART), in 1996 (Cooper and Merigan 1996). HAART permits to maintain low viral load in HIV-1 positive patients, resulting in two effects: 1) it prevents AIDS-related infections and patients' death and 2) it decreases the risk of HIV-1 transmission. The HAART therapy is a combination of more than three drugs belonging to different antiretroviral classes. This permits a reduction of the insurgence of drug resistances in the treated patients. Different factors must be considered in the choice of combination drugs, as viral load, $CD4^+$ T-cells count, age, gender,

HIV-1 drug resistance, co-infections and concomitant other medical treatments. To achieve a high compliance some medicines are available combined together in one pill.

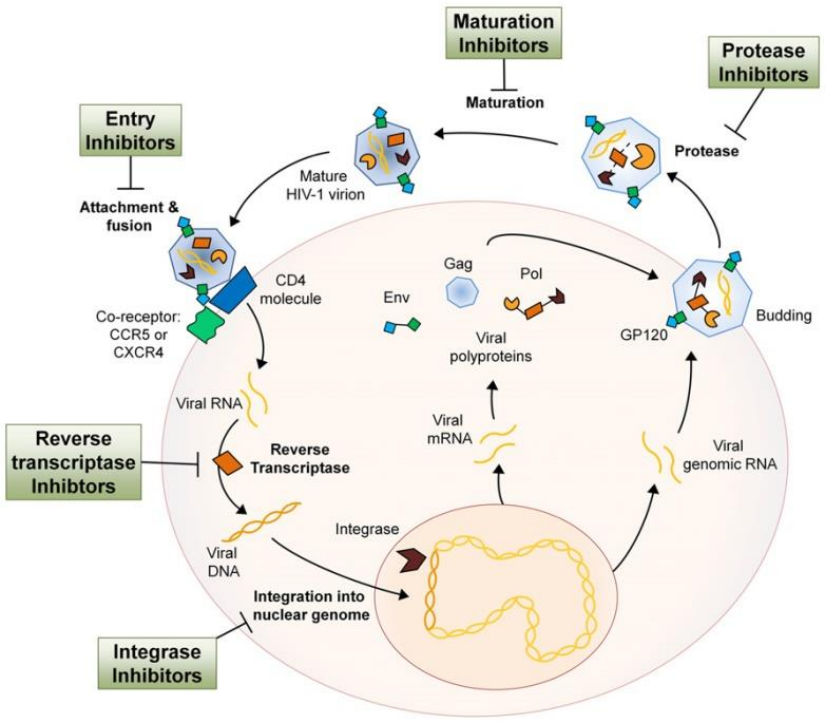


Figure 8. Antiretroviral drug class intervention points.
The five classes of HAART drugs are shown below: nucleoside/nucleotide Reverse Transcriptase inhibitors (NRTIs), Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease inhibitors, Fusion/Entry inhibitors, Integrase inhibitors (Smith, de Boer et al. 2012).

HAART introduction has improved the length and the life quality of HIV-1 infected individuals, reducing viral transmission in Europe, North America and other Western countries. While, at the beginning of the 2000's in African Countries a health response to the HIV-1 epidemic did not yet exist, today, the response toward HIV-1 emergency is bringing treatment and delivering life-saving services to remote African communities (Source: Global AIDS Response Progress Reporting UNAIDS/UNICEF/WHO and UNAIDS/WHO estimates) (Figure 9).

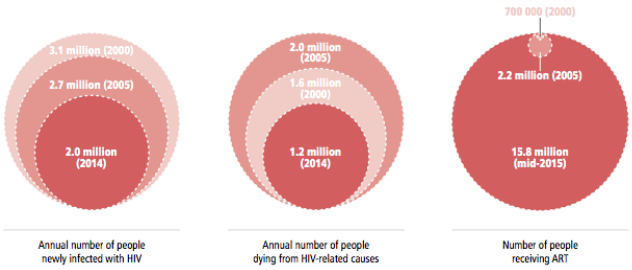


Figure 9. Progress in the global HIV-1 response in African Regions in the years 2010-2015.
Source: Global AIDS Response Progress Reporting UNAIDS/UNICEF/WHO and UNAIDS/WHO estimates.

Since HAART introduction, many new drugs have been developed in response to problems of toxicity, tolerability and resistance, and great improvements have been achieved in accessibility of HIV drugs in resource-poor global regions (Looney, Ma et al. 2015). But unfortunately, the incidence of drug resistance viruses is increasing, and significant problems caused by long-term toxicity of drug treatments are reported among treated people (Reust 2011). As a consequence, the HIV-1 eradication remains a difficult aim to be achieved, making necessary the discovery of new effective drugs. Furthermore, important efforts have been made to reduce the risk of vertical and horizontal transmissions encouraging preventive measures through education and public information. One way to reduce transmission is to recommend application of topical microbicide formulations and condom use. Male circumcision could be an additional efficient strategy to reduce the risk of sexual transmission of HIV-1 (Shattock and Moore 2003).

While antiretroviral drugs are able to control AIDS pathogenesis and to prevent HIV-1 spread, the development of a vaccine against HIV-1 would represent an important goal toward the eradication of the infection. Although there are no effective cures at present, the efforts to develop virus eradication strategies are not extinguished yet, encouraged by the “Berlin patient”. Berlin patient is Timothy Ray Brown, which was diagnosed with HIV in 1995 and began antiretroviral therapy. In 2006, he was diagnosed with acute myeloid leukemia (AML) and in 2008, as AML cure he received a bone marrow transplantation from a donor with an homozygous CCR5Δ32 mutation, which confers resistance to HIV-1 infection (Hutter, Nowak et al. 2009). Since that moment he has maintained low levels of

HIV and has remained off HAART therapy. It is now known that the CCR5Δ32 variant represents an HIV-1 resistance factor, since it avoids the HIV entry (Allers, Hutter et al. 2011). This case demonstrates the critical role that CCR5 plays in maintaining HIV-1 infection (Hutter, Nowak et al. 2009). Thus, different strategies targeting CCR5 have been employed to counteract HIV-1 infection progression (Figure 10). Maraviroc, a CCR5 antagonist was approved as anti HIV-1 drug by FDA in 2007 (Woollard and Kanmogne 2015).

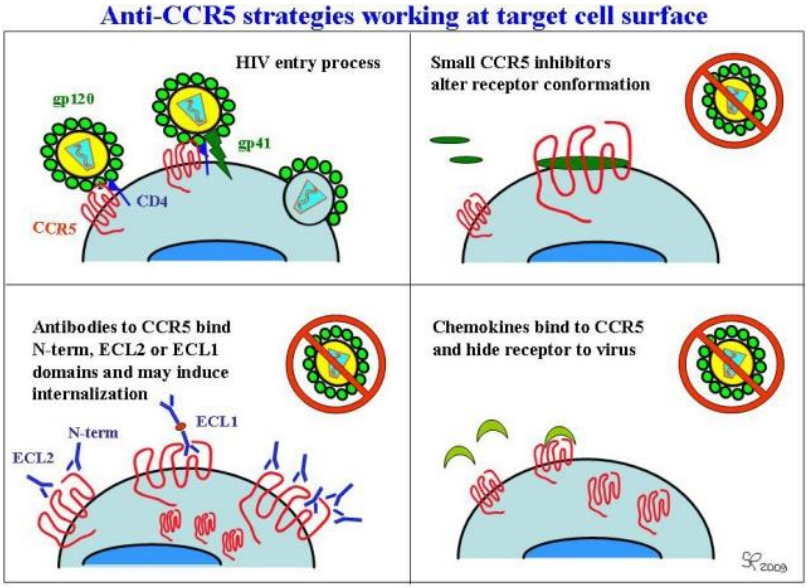


Figure 10. Current approaches to extracellular CCR5 blocking.
The HIV-1/CCR5 binding can be blocked using: CCR5 inhibitors which alter the receptor conformation, antibodies against CCR5 domains recognized by HIV-1, chemokines which bind CCR5 hiding the receptor to HIV-1 (Lopalco 2010).

HIV vaccine: state of art

Despite the great efforts to develop an effective HIV vaccine, the quest for a safe and successful HIV vaccine seems to be remarkably long and difficult.

Efficient vaccines normally stimulate protective immunity similar to that which occurs during a natural infection (Cohen and Dolin 2013). The mechanism whereby an HIV vaccine confer protection remains uncertain, and an effective vaccine may require stimulation of an immune response that is considerably different from that observed during natural infection (Johnston and Fauci 2011).

The extreme HIV diversity is another challenge to vaccine design, particularly the diversity of HIV-1 is surprising (Hemelaar 2012). An immunogen derived from a particular clade may thus be ineffective against other clades, making the development of a global HIV vaccine extremely difficult.

Before the development of protein subunit vaccines, both attenuated and inactivated vaccines had been tested, without success, in nonhuman primates (NHPs) (Girard, Osmanov et al. 2011). Protein subunit vaccines for HIV are centred on the HIV envelope. The mature envelope is made by a trimer, composed of three gp120/gp41 complexes. Both recombinant gp120 (rgp120) and gp160 (rgp160) monomers were identified as potential immunogens in early HIV vaccine strategies (Dolin, Graham et al. 1991, Keefer, Graham et al. 1994). An rgp120 immunogen derived from MN HIV-1 strain showed protection against heterologous strains in chimpanzees (Berman, Murthy et al. 1996), and it proved safe and immunogenic in humans (Migasena, Suntharasamai et al. 2000). This immunogen is the basis for the VAX004 and VAX003 trials. These trials demonstrated that rgp120 monomers stimulated partial neutralizing antibody responses and failed to confer protection against HIV infection in high-risk populations (Figure 11) (Flynn, Forthal et al. 2005, Pitisuttithum, Gilbert et al. 2006).

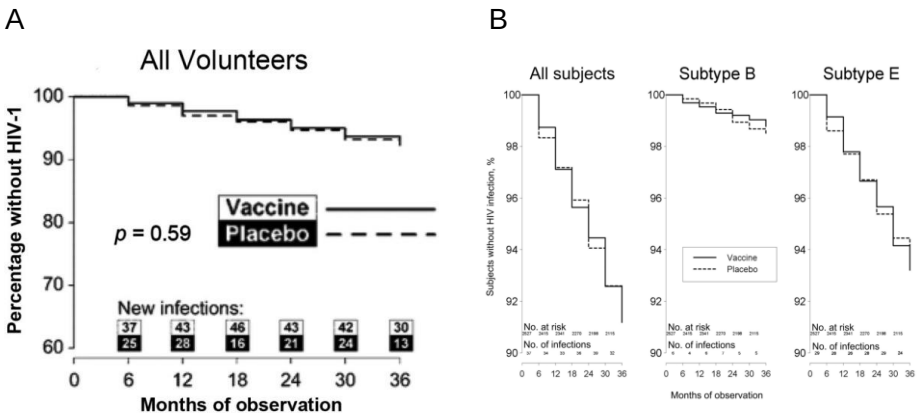


Figure 11. Survival curves of HIV-1 infected patients enrolled in VAX004 and VAX003 trials. Kaplan–Meier curves from VAX004 (A) and VAX003 (B) displaying time to HIV-1 infection. Adapted from (Flynn, Forthal et al. 2005, Pitisuttithum, Gilbert et al. 2006).

A recent major advance in the development of an effective vaccine against HIV-1 has been the detection of highly potent broadly neutralizing antibodies (bnAbs). It was reported that roughly 20% of infected individuals presented bNAbs, able to neutralize a wide range of HIV-1 viral isolates, after 2-3 years of infection. However, unlike typical viral infection, these bNAbs are not able to control the viral replication due to the emergence of escape variants (Sather, Armann et al. 2009, Gray, Madiga et al. 2011). Most bNAbs belong to the following groups on the bases of the epitopes that they recognize: CD4 binding site, V1/V2 variable loops, exposed glycans, and proximal external region membrane (Figure 12).

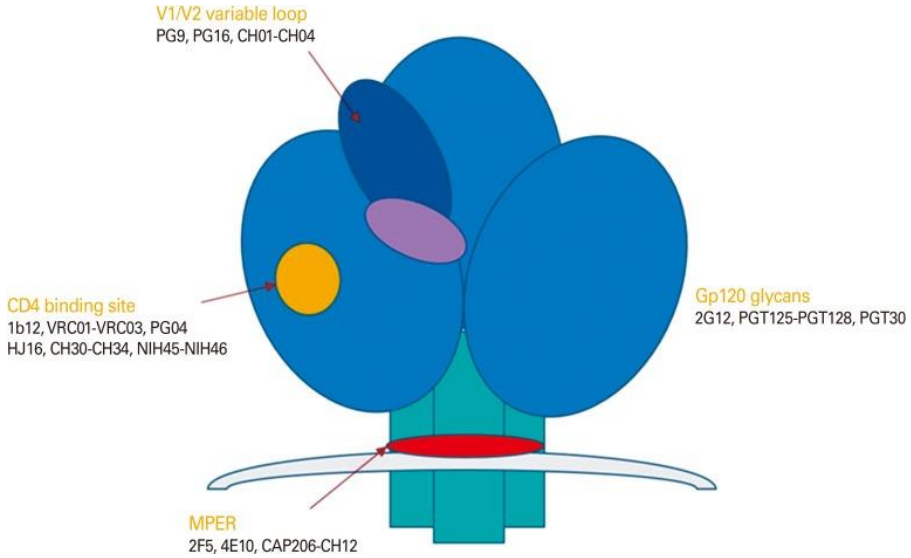


Figure 12. Schematic drawing of HIV-1 Env trimer with highlighted epitopes for broadly neutralizing antibodies. The known four general specificity for broadly neutralizing antibodies detected are the membrane proximal external region (MPER), the CD4 binding site, the V1/V2 variable loops and certain exposed glycans. Red: MPER of gp41, Blue: gp120 core, dark blue: V1/V2 loops, magenta: V3 loop, green: gp41, light gray: viral membrane bilayer (Shin 2016).

The bNAbs efficacy in preventing infection is not yet proven in humans, but nonhuman primate studies with passive transfer of bNAbs showed promising results (Barouch, Whitney et al. 2013, Shingai, Nishimura et al. 2013). Human *in vivo* study has shown decreased levels of circulating virus after treatment of bNAbs (Caskey, Klein et al. 2015).

The development of HIV Env neutralizing antibodies is mostly hampered by: 1) the remarkable antigenic diversity of HIV-1 Env, 2) the hidden epitope by the quaternary structure and glycosylation, 3) antibodies must undergo extensive somatic hypermutation to gain the ability to recognize the native trimer (Klein,

Mouquet et al. 2013, Mascola and Haynes 2013). To solve these problems, the envelop trimer has been stabilized in a soluble form and used as immunogen (Chen, Kovacs et al. 2015, Dosenovic, von Boehmer et al. 2015, Jardine, Ota et al. 2015), but the development of a pure stable envelope immunogen capable to mimic the functional envelope spike remains a big challenge (Shin 2016).

Another vaccine approach exploits the engagement of the naïve B cell repertoire. This approach permits the identification of the drivers that are responsible for the sequential stimulation of HIV-1 reactive B cell lineage to harvest the bNAbs and use the information to create templates for designing immunogens (Shin 2016).

Disappointing results in stimulating B cells, made HIV researchers to turn from B cell targeted vaccines intended to induce neutralizing antibodies, to T cell targeted approach. Anyway, the CTL vaccine purpose is to decrease the viral set point and delay disease progression, somewhat than to prevent initial infection (Fauci and Marston 2015).

Although our comprehension of viral immunology and immune correlate of protection has improved remarkably during the years, the quest to develop an effective HIV vaccine is long and winding (Shin 2016). Future progresses will depend on an iterative relationship between findings from preclinical studies and from appropriately designed, efficiently conducted clinical trials (Cohen and Dolin 2013).

MHC I: HLA-A, HLA-B and HLA-C

The MHC Class I genes are found in all vertebrates, although they present a high variability. In humans, the MHC region is located on chromosome 6. It consists of 19 gene loci: three classical (called HLA-A, HLA-B and HLA-C), three non-classical (known as HLA-E, HLA-F and HLA-G) and 12 referred as non-coding genes or pseudo-genes. The MHC-I proteins are expressed on all the cell membranes.

The classical MHC-I genes differ from the non-classical ones since they are highly polymorphic. Among all, HLA-B is the most polymorphic locus in the

human genome, followed by HLA-A and HLA-C (Davidson, Kress et al. 1985). The MHC-I complexes are heterodimers composed of a membrane-bound heavy chain (encoded by the HLA-A, HLA-B or HLA-C gene), not covalently associated to an invariant light chain, called β_2m. MHC-I complexes bind short peptides (8-11 aminoacids), derived from the degradation of intracellular proteins and present these antigens to $CD8^+$ T-cells. $CD8^+$ T-cells are able to kill the cells displaying pathogen antigens on their MHC-I molecules ensuring the elimination of infected cells from the organism (Groothuis and Neefjes 2005). In addition MHC-I molecules control NK cells responses via interaction with their Killer KIR, leading to inhibition or activation of their cytolytic function. HLA-C presents antigen to CTLs less efficiently than HLA-A and HLA-B do (Falk and Schendel 1997), but it is an extremely good ligand for inhibitory KIR receptors, protecting target cells from NK cells mediated lysis (Colonna, Borsellino et al. 1993).

HLA-C expression level

It is known that HLA-C on the cell surface is expressed at lower level than HLA-A and HLA-B. Several mechanisms are involved in the regulation of HLA-C cell surface expression:
1) the HLA-C molecules have a poor assembling efficiency with the light chain, β_2m, which determines an intracellular accumulation as free chains in the ER, followed by their degradation (Sibilio, Martayan et al. 2008) and more importantly, HLA-C show a more selective peptide-binding characterized by a low affinity constant compared to HLA-A and HLA-B (Neisig, Melief et al. 1998). 2) HLA-C has a di-hydrophobic motif (L336-I337) in the cytoplasmic tail that causes a more rapid internalization than HLA-A and HLA-B (Schaefer, Wonderlich et al. 2008, Kulpa and Collins 2011). 3) A subset of HLA-C allotypes presents in the 3'UTR of its mRNA a target sequence for a microRNA (called Hsa-miR-148a) able to interfere at post-transcriptional level with HLA-C gene expression (Kulkarni, Savan et al. 2011) (Figure 13).

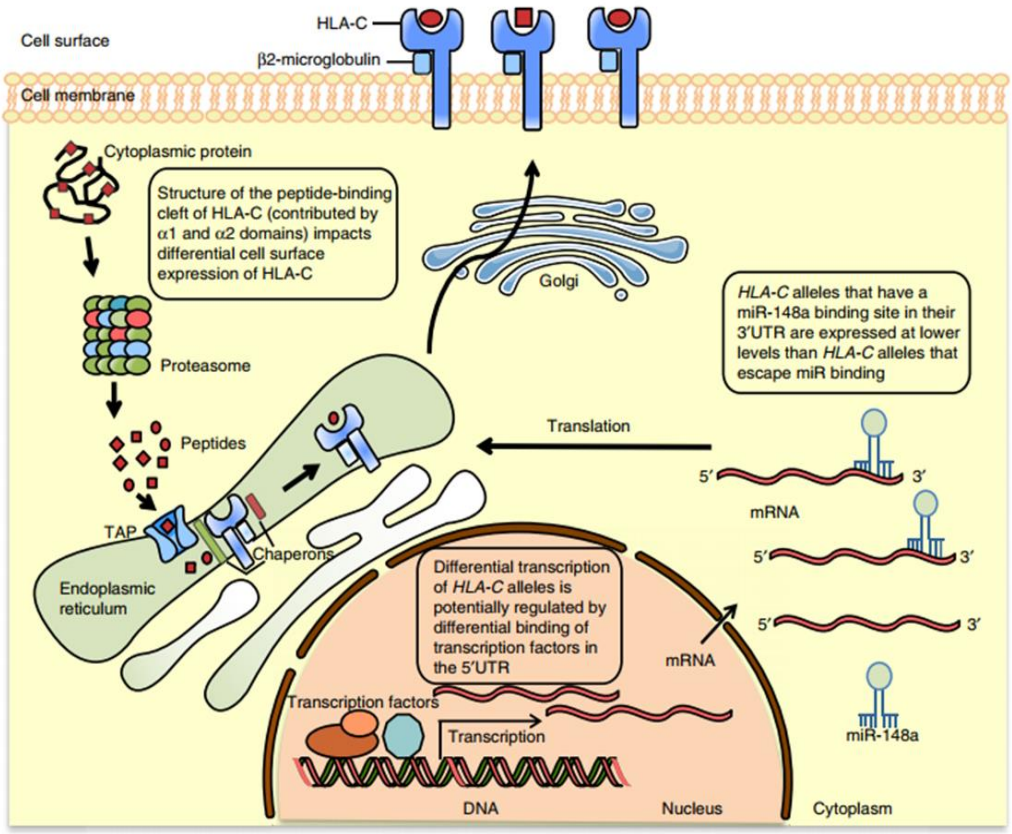


Figure 13. Regulation of HLA-C surface expression.
The HLA-C surface expression depends on different variants present in 5' and 3' UTR, variation in the antigen-binding cleft, and possibly other yet unidentified factors (Kaur, Gras et al. 2017).

HLA-C role in HIV-1 infection

A genome wide association study (GWAS) identified a Single Nucleotide Polymorphism (SNP) located 35 kb upstream of the HLA-C coding sequence (rs9264942), associated with different HLA-C expression levels, as a major genetic determinant for HIV-1 host control (Fellay, Shianna et al. 2007). Subsequently, the causal variant responsible for these associations was identified in another SNP (rs67384697) that maps in the 3' UTR mRNA of HLA-C alleles, which affects the binding of the microRNA Hsa-miR-148a (Kulkarni, Savan et al. 2011). This SNP was shown to partially influence cell surface expression of HLA-C with poor expressing alleles, such as HLA-C*01, C*03, C*04 and C*07, that maintain an intact miR148a binding site (263G) and high expressing alleles, such as HLA-C*02, C*05, C*06, C*08, C*12, C*15, C*16, in which the site is

deleted (263del), thus escaping the regulation by interference played by that microRNA (Kulkarni, Savan et al. 2011). Among all the subset of HLA-C alleles that are inhibited by miR-148a, differential expression levels of miR-148a itself (SNP rs735316) contribute to variable levels of HLA-C expression. This contribution is relevant among subjects who have at least one copy of HLA-C allele inhibited by miR-148a, while there is no detectable effect among subjects carrying two escape HLA-C variants (Kulkarni, Savan et al. 2011). Vince et al. recently characterized a new SNP, rs2395471, located in the HLA-C promoter region, associated with variable HLA-C expression levels both in European and African Americans (Kulkarni, Savan et al. 2011, Vince, Li et al. 2016).

An HLA-C increased expression was associated with delayed progression toward AIDS in both European Americans and Africans, regardless of their different HLA-C frequencies and linkage relationships with HLA-B and HLA-A (Kulkarni, Savan et al. 2011).

Adding complexity to this matter, other studies failed to confirm the association between HLA-C expression and these genetic markers (Corrah, Goonetilleke et al. 2011, Gentle, Paximadis et al. 2013, Bettens, Buhler et al. 2016) and a study conducted on a specific population in Nairobi (Sampathkumar, Peters et al. 2014) reported that the same HLA-C allotype, C*07, could be associated either with a slow rate of seroconversion (C*07:01) and with an increased rate of seroconversion (C*07:02). Furthermore, it has been reported that highly expressed HLA-C alleles are associated to an increased risk of Chron's Disease, while the low expressed ones are associated to a lower risk of Chron's Disease (Kulkarni, Qi et al. 2013) (Figure 14). It has been hypothesized that higher HLA-C expression levels might induce HIV-1-specific responses through either binding to KIRs on NK cells and increasing antigen presentation to CTLs (Apps, Qi et al. 2013) or a combination of these mechanisms, explaining the slower progression toward AIDS (Malnati, Ugolotti et al. 2017). At the same time the higher HLA-C expression could, through molecular mimicry, activate autoreactive T cells and acting as superantigens that stimulate a large number of T cells causing an increased risk of Chron's disease (Moller 1998).

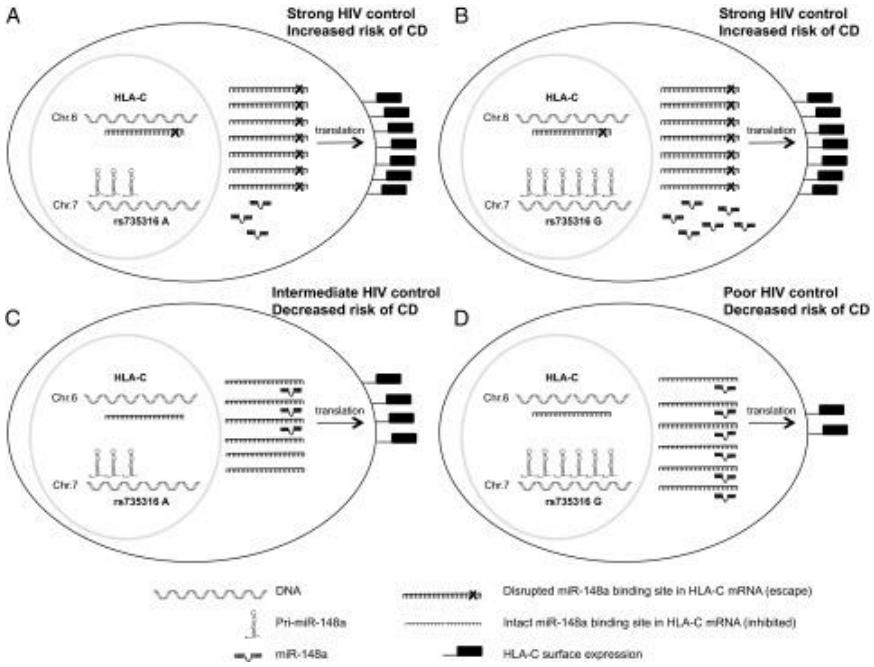


Figure 14. Double steps regulation of HLA-C expression.
Expression levels of HLA-C escape alleles which have a disrupted miR-148a binding site (x) are not influenced by the MIR148A genotype (A or B). HLA-C expression levels of alleles which have an intact miR-148a binding site are inhibited by miR-148a in a dose-dependent manner (C and D) (Kulkarni, Qi et al. 2013).

Env/HLA-C

It has been shown that during the budding process from the cell membrane, MHC-I and MHC-II molecules are incorporated into the nascent HIV-1 virions together with other cellular proteins, with a higher number of MHC molecules than Env trimers (Zhu, Udaka et al. 2006, Lakhashe, Tripathy et al. 2008). It has been reported that this proteins incorporation is not dependent on the relative proteins amount on the infected cell surface, since some highly expressed proteins such as CD45, CD4, CCR3, CCR5 or CXCR4 are not incorporated into the nascent virions (Esser, Bess et al. 2001). MHC-I free chains are able to cis-associate with themselves as well as with a wide membrane receptors variety, such as CD3, CD8αβ, CD25 and IL-15Rα (Tremblay, Fortin et al. 1998, Esser, Bess et al. 2001, Arosa, Santos et al. 2007, Ott 2008). HLA-C has a specific aptitude to associate with viral proteins, in particular with HIV-1 Env, since its weak bound with the β_2m compared to the one of HLA-A and HLA-B, and it can

easily accumulate as HLA-C free chains on the cell surface.

MHC-I negative cell lines are non-permissive for the HIV-1 primary isolates replication (Cosma, Blanc et al. 1999), but HLA-C transfection in these cells recovers HIV-1 replication competence. HLA-C might be involved in inducing changes in viral envelope protein conformation, and in enhancing presentation of epitopes normally exposed upon CD4 binding (Cosma, Blanc et al. 1999). Moreover, HLA-C incorporation in virions has been shown to reduce susceptibility to neutralizing antibodies thus promoting the progression of the disease (Cosma, Blanc et al. 1999). A specific, non-covalent association between HLA-C free chains and gp120, within CD4-CCR5-gp120/gp41 fusion complexes, forming on cells during the process of cell-to-cell fusion induced by HIV-1 was documented (Matucci, Rossolillo et al. 2008). In the same study it was reported that fusion efficiency is reduced in HLA-C negative cells and that viruses produced in HLA-C silenced cells present a significantly lower infectivity than those produced in HLA-C expressing cells (Matucci, Rossolillo et al. 2008). Further studies demonstrated that HLA-C is selectively incorporated into the HIV-1 virions, associating with the viral glycoprotein Env and modulating viral infectivity (Baroni, Matucci et al. 2010, Zipeto and Beretta 2012).

The protective role of high levels of HLA-C expression in HIV-1 infection is in apparent contrast with results that suggest a role for the Env/HLA-C association in increasing viral infectivity. This apparent contradiction may be due to the presence of different HLA-C conformations and their relative amounts. HLA-C, in fact, could be associated either with the β_2m (its physiological partner) or with HIV-1 Env (Matucci, Rossolillo et al. 2008, Sibilio, Martayan et al. 2008).

HLA-C/β_2m/peptide binding stability

Sibilio *et al*. showed that differences in binding stability to β_2m do exist between different HLA-C alleles (Sibilio, Martayan et al. 2008). Some of them are preferentially present as free chains due to their low binding stability to β_2m, while other are preferentially present as full complex, consisting of the heavy HLA-C chain bound to β_2m/peptide. Interestingly, some HLA-C alleles with a lower binding stability to β_2m are also those described as low expressing alleles (Kulkarni, Savan et al. 2011) (C*01, C*03, C*04, C*07) while some alleles with a higher binding stability are also those described as high expressing alleles (C*02, C*05, C*06, C*08).

In a recent study, Kaur and colleagues (Kaur, Gras et al. 2017) reported that differences in HLA-C sequences in exons 2-3 (which encode the α1/α2 domains) drive differential expression of HLA-C allomorphs at the cell surface by influencing the structure of the peptide-binding cleft and the diversity of peptides bound by the HLA-C molecules. Specifically, they demonstrated that the peptide-binding cleft of HLA-C*05 is more permissive and is filled with large aromatic residues, which is not the case for HLA-C*07. Instead of forming a groove as in HLA-C*07, the peptide-binding cleft of HLA-C*05 forms a flatter ‘peptide-landing platform’, that allows binding of a larger range of peptides, which can stabilize the HLA-C molecule, in turn affecting its expression levels on the cell surface.

CRISPR/Cas9 Introduction

In the present study I investigated the HLA-C role in HIV-1 infection. It is known that the β_2m protein, is required for the HLA molecules translocation to the membrane cells, where HLA-C interacts with HIV-1 Env modulating viral infectivity (Zipeto and Beretta 2012). Thus, I used the CRISPR/Cas9 technique to inactivate the β_2m gene in HEK-293T, HeLa-Lai (expressing HIV-1 Env), and parental HeLa cells. Since the CRISPR/Cas9 technique is relatively recent, a short introduction on this system will help to better understand how it works.

Genome editing and CRISPR/Cas system

Genome editing permits to selectively modify DNA sequences in the cell genome. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system exploits the cellular repair mechanisms, activated by the presence of a Double Strand Break (DSB) introduced in the cell genome by the Cas9 endonuclease. DSBs could be repaired by one of at least two different pathways: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Figure 15). NHEJ leads to the introduction of insertion/deletion mutations (indels), which thus disrupt the correct translational reading frame (Bibikova, Carroll et al. 2001). Instead, HDR-mediated repair can be exploited to insert specific desired sequences through recombination of the target locus with exogenously supplied DNA (Sander and Joung 2014).

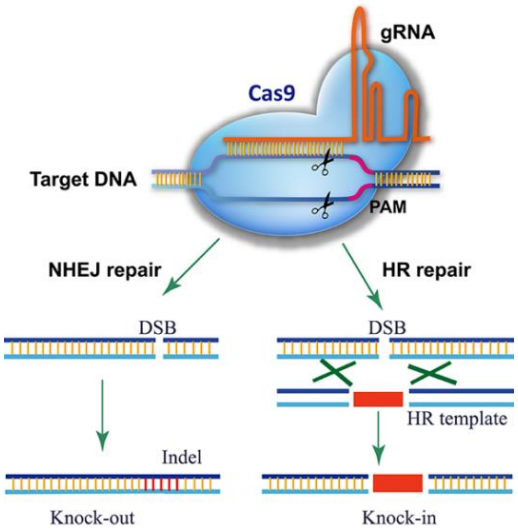


Figure 15. Scheme of Cas9/gRNA genome editing. Cas9 cuts both strands of the target DNA thanks to the gRNA. The DSB could be repaired via the error-prone NHEJ pathway, or via the HDR pathway (Ding, Li et al. 2016).

CRISPR/Cas9 system

The most used Cas9 endonuclease in genome editing is the *Streptococcus pyogenes* (SpCas9) derived one. A human codon-optimized Cas9 protein bearing the SV40 nuclear localization signal at C-terminal was developed to make it suitable for genome engineering in eukaryotic cells (Shen, Brown et al. 2017). To guide the Cas9 endonuclease to a specific DNA target it is exploited an RNA guide, called sgRNA (Mali, Yang et al. 2013). For the Cas9 specific-cleavage, in addition to the sgRNA/target DNA complementarity, it is required the presence of a Protospacer Adjacent Motif (PAM), a 3 bp sequence flanking the 3' end of the DNA target site (Jinek, Chylinski et al. 2012) (Figure 16).

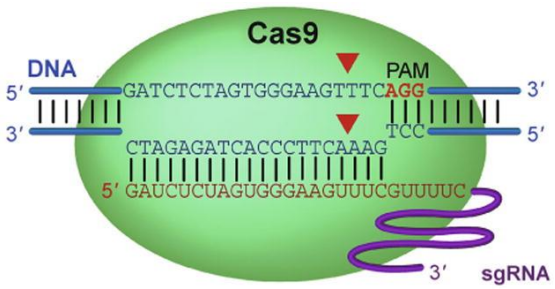


Figure 16. Cas9/DNA complex.
In green the Cas9 endonuclease, in purple the sgRNA, in red the PAM sequence (Wu, Kriz et al. 2014).

Aim of the thesis

The effective role of the HLA-C/HIV-1 Env interaction is still not fully clear. The aim of this thesis was to characterize the association between HLA-C and HIV-1 Env through flow cytometry experiments and co-immunoprecipitation assays to clarify its implication in HIV-1 infection. In addition, using the CRISPR/Cas9 technique, I developed β_2m negative cell lines, to investigate the role of HLA-C in modulating HIV-1 infectivity.

Some studies reported that the HLA-C presence on HIV-1 virions enhances their infectivity (Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010), suggesting a role for the HIV-1 Env/HLA-C association in increasing HIV-1 infectivity.

On the contrary, other studies reported an HLA-C protective role in HIV-1 infection when it is expressed at high levels on the cell surface (Kulkarni, Savan et al. 2011, Apps, Qi et al. 2013).

This apparent contradiction may be due to the presence of different HLA-C conformations, free chains or HLA-C/β_2m/peptide immunocompetent trimers (Sibilio, Martayan et al. 2008, Serena, Parolini et al. 2017). Thus, differences among HLA-C allotypes in modulating HIV-1 infectivity could rely on the intrinsic different stability as heterotrimeric complexes.

To understand the basis of these observations, I deeply investigated HLA-C surface reactivity by comparing two different antibodies, DT9 and L31, specific for HLA-C/β_2m/peptide heterotrimers and HLA-C free chains respectively.

Secondary was tested on a limited panel of isolates if HLA-C stability could affect the HIV-1 infectivity.

Results

HIV-1 infection induces HLA-C free chains surface expression in infected cells

To test the effects of HIV-1 infection on HLA-C surface expression, two cell lines were employed: A3.01 and ACH-2. A3.01 is a T-lymphocytic cell line and ACH-2 represents the HIV-1 chronically infected counterpart (Clouse, Powell et al. 1989). TNF-α stimulation is associated with the induction of nuclear factors binding to the NF-kB site in the HIV-1 LTR region, leading to viral reactivation (Duh, Maury et al. 1989). Both A3.01 and ACH-2 cells were stimulated with TNF-α for 24 hours and analysed by flow cytometry for gp120 HIV-1 Env glycoprotein (using 2G12 antibody) and the HLA-C expression. HLA-C expression was assessed using two different antibodies: the DT9 antibody which recognizes the heterotrimeric complexes HLA-C/β_2m/peptide (Braud, Allan et al. 1998) and the L31 monoclonal antibody, specific for HLA-C free chains (Setini, Beretta et al. 1996). As expected, following TNF-α stimulation, an increase of 2G12 reactivity was observed in ACH-2 cells, confirming the viral reactivation. Upon TNF-α stimulation, an increase of HLA-C/β_2m/peptide (DT9 reactivity) was detected in both cell lines (A3.01 and ACH-2). The upregulation of HLA-C heterotrimeric complexes is due to the TNF-α activity in modulating MHC-I expression (Hallermalm, Seki et al. 2001). Interestingly, upregulation of HLA-C free chains (L31 reactivity) was observed only in the ACH-2 cells (Figure 17). As reported in Figure 17, L31 reactivity in ACH-2 cells, was significantly different between unstimulated and TNF-α stimulated cells (two-way ANOVA, $p = 0.002$), as well as towards the TNF-α stimulated A3.01 cells (two-way ANOVA, $p = 0.01$).

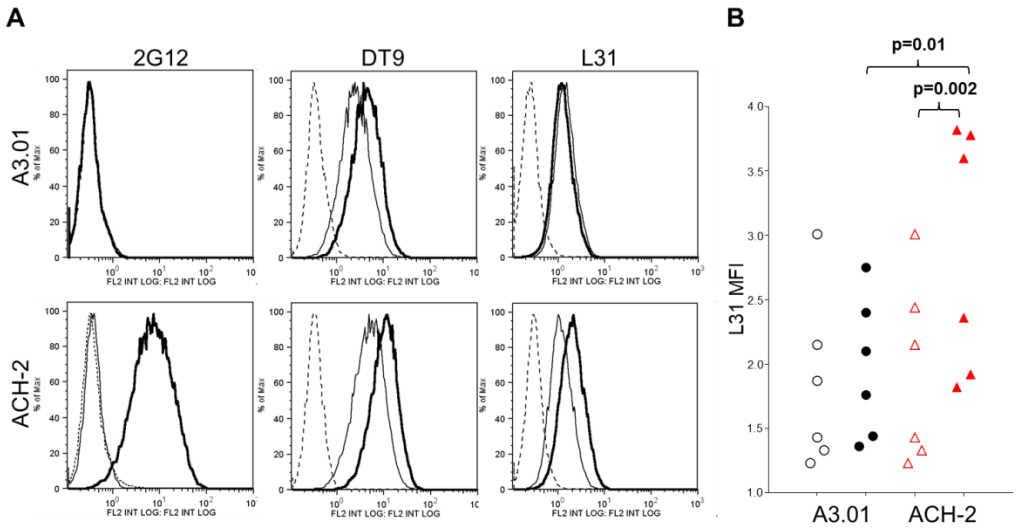


Figure 17. Flow cytometry analysis of HLA-C and Env surface expressions in A3.01 and ACH-2 cells.
A. Dashed line: secondary antibody control; thin line: unstimulated cells; thick line: 24 hours TNF-α stimulated cells. 2G12, DT9 and L31 antibodies recognize: HIV-1 Env, HLA-C heterotrimeric complexes and HLA-C free chains respectively. Upon TNF-α stimulation, ACH-2 cells express HIV-1 Env (2G12); HLA-C heterotrimers (DT9) are upregulated in both cell lines; HLA-C free chains (L31) are upregulated in ACH-2 cells only.
B. Empty black dot ○: Unstimulated A3.01 cells; Full black dot ●: TNF-α stimulated A3.01 cells; Empty red triangle △: Unstimulated ACH-2 cells; Full red triangle ▲: TNF-α stimulated ACH-2 cells. Experiment was repeated 6 times, median fluorescence intensity (MFI) of L31 antibody are displayed. HLA-C free chains surface expression is significantly different between TNF-α stimulated ACH-2 cells (chronically infected by HIV-1) and A3.01 cells (uninfected parental cell line) ($p = 0.01$), and between TNF-α stimulated and unstimulated ACH-2 cells ($p = 0.002$). p values refer to two-way ANOVA analyses. Adapted from: (Serena, Parolini et al. 2017).

A time course analysis of HIV-1 Env, MHC-I and β_2m expressions in A3.01 and ACH-2 cells was performed. Both cell types were analysed by flow cytometry comparing the antibody fluorescence with and without TNF-α stimulation after 24, 48 and 72 hours from stimulation. The membrane expression of HLA-C free chains (L31 reactivity) correlated with HIV-1 Env expression (2G12 reactivity), and both decrease at later times. On the contrary, the total HLA-C expression (detected with the DT9 antibody and with the L31 antibody after acid wash treatment), the MHC-I (detected with the W6/32 antibody) and the β_2m (detected with NAMB-1 antibody) expressions showed no differences (Figure 18).

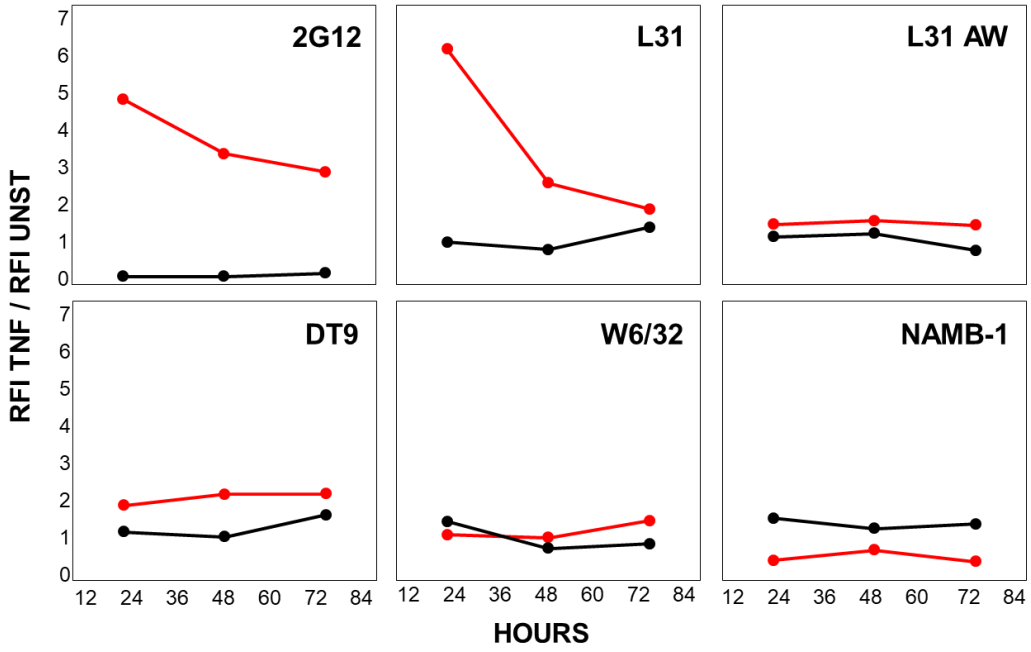


Figure 18. Time course analysis of HIV-1 Env, MHC-I and β_2m surface expressions, in TNF-α stimulated and unstimulated A3.01 and ACH-2 cells.
Cells were surface labelled at different times, 24, 48 and 72 hours (X-axis). Red line: ACH-2 cells; black line: A3.01 cells. Fluorescence fold change calculated as the ratio between RFI of TNF-α stimulated and unstimulated cells are reported on Y-axis. Of note, the correlation between the expression of Env and HLA-C free chains: the HLA-C free chains surface expression appears to be dependent on the presence of HIV-1 Env. No differences in the expression of total HLA-C free chains after acid wash (L31 AW panel), HLA-C trimeric complex (DT9 panel), MHC-I (W6/32 panel) or β_2m (NAMB-1 panel) are observed. Adapted from: (Serena, Parolini et al. 2017).

The correlation, between HIV-1 infection and HLA-C free chains surface expression was further confirmed in an additional cellular model. PM1 cells, a human T-lymphocytic cell line chronically infected with HIV-1 IIIB, was used (Figure 19A). The flow cytometry analyses of HIV-1 Env (2G12) and MHC-I molecules (W6/32) showed a constitutive basal viral production (without TNF-α stimulation) higher than the one observed in ACH-2 cells. As previously observed in ACH-2 cells, after 48 hours of TNF-α stimulation in PM1-IIIB cells, an increase of HLA-C free chains at the cell surface (L31 reactivity) was observed. This increase was hardly detected in the uninfected counterpart PM1 cells. It could be speculated that HIV-1 infection promotes the formation of HLA-C free chains at the membrane of infected cells. Noteworthy in PM1-IIIB cells, the TNF-α stimulation counteracted the HIV-1 induced MHC-I downregulation.

Having established the correlation between HLA-C free chains appearance and HIV-1 infection in both used cellular models, we further investigated which component of HIV-1 was involved in this phenomenon, starting from the HIV-1 Env protein. Thus, HEK-293T cells were transfected with a plasmid encoding HIV-1 QHO-Env and a plasmid encoding an Env defective full-length HIV-1 genome named pSG3$^{\Delta env}$ (Figure 19B). An increase of L31 reactivity was detected only after QHO-Env transfection. After acid wash treatment, no differences in L31 reactivity were detected. Probably HIV-1 Env protein induces a switch from HLA-C heterotrimeric complex to HLA-C free chain.

HLA-C free chains surface appearance is not due to any HIV-1 proteins

To explore if any other HIV-1 proteins were responsible for the appearance of HLA-C free chains on the cell surface, plasmids encoding different HIV-1 proteins were transfected in β_2m knockout HEK-293T cells, generated using the CRISPR/Cas9 system. Different viral proteins were tested: Gag, Env, Vpu, Vif, Nef, Tat. In addition, the Env defective full-length HIV-1 genome (pSG3$^{\Delta env}$) plasmid was used. After transfection, HEK-293T cells were treated with the acid wash to displace the β_2m and then analysed by flow cytometry using the L31 antibody, to exclude that the potential intracellular association between HLA-C/viral protein might mask the L31 epitope (Setini, Beretta et al. 1996). Only the transfection with the control β_2m expressing vector restored the HLA-C surface expression, while no HLA-C expression was detected on the cell membrane after transfection with the other plasmids, expressing HIV-1 proteins (Figure 20).

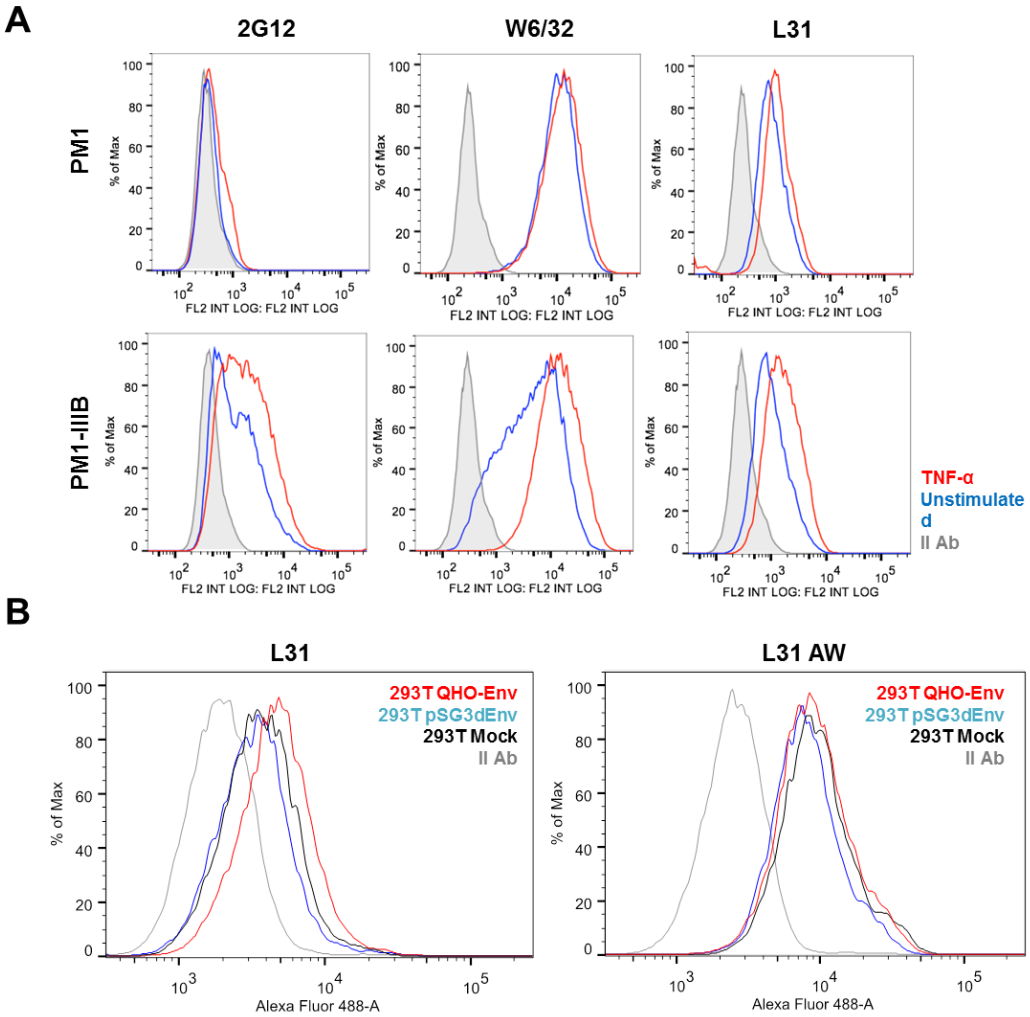


Figure 19. HIV-1 Env expression and HLA-C free chains surface expression.
A. Shaded curve: Secondary antibody control; blue line: Unstimulated cells; red line: Cells stimulated for 48 hours with TNF-α. PM1 cells infected with HIV-1 IIIB showed a downregulation of MHC-I molecules (W6/32) and a slight HLA-C free chains increase (L31).
Upon TNF- α stimulation, and thus the viral reactivation, the MHC-I surface expression was restored.
B. Grey line: Secondary antibody control; black line: HEK-293T transfected with pcDNA3; blue line: HEK-293T transfected with pSG3$^{\Delta env}$; red line: HEK-293T transfected with HIV-1 QHO-Env plasmid. In HEK-293T transfected with HIV-1 Env was observed an increase of HLA-C free chains (L31) at the cell membrane compared to the HEK-293T transfected with pcDNA3 (mock) or pSG3$^{\Delta env}$ plasmids. After acid wash (L31 AW) the total HLA-C molecules were comparable (Serena, Parolini et al. 2017).

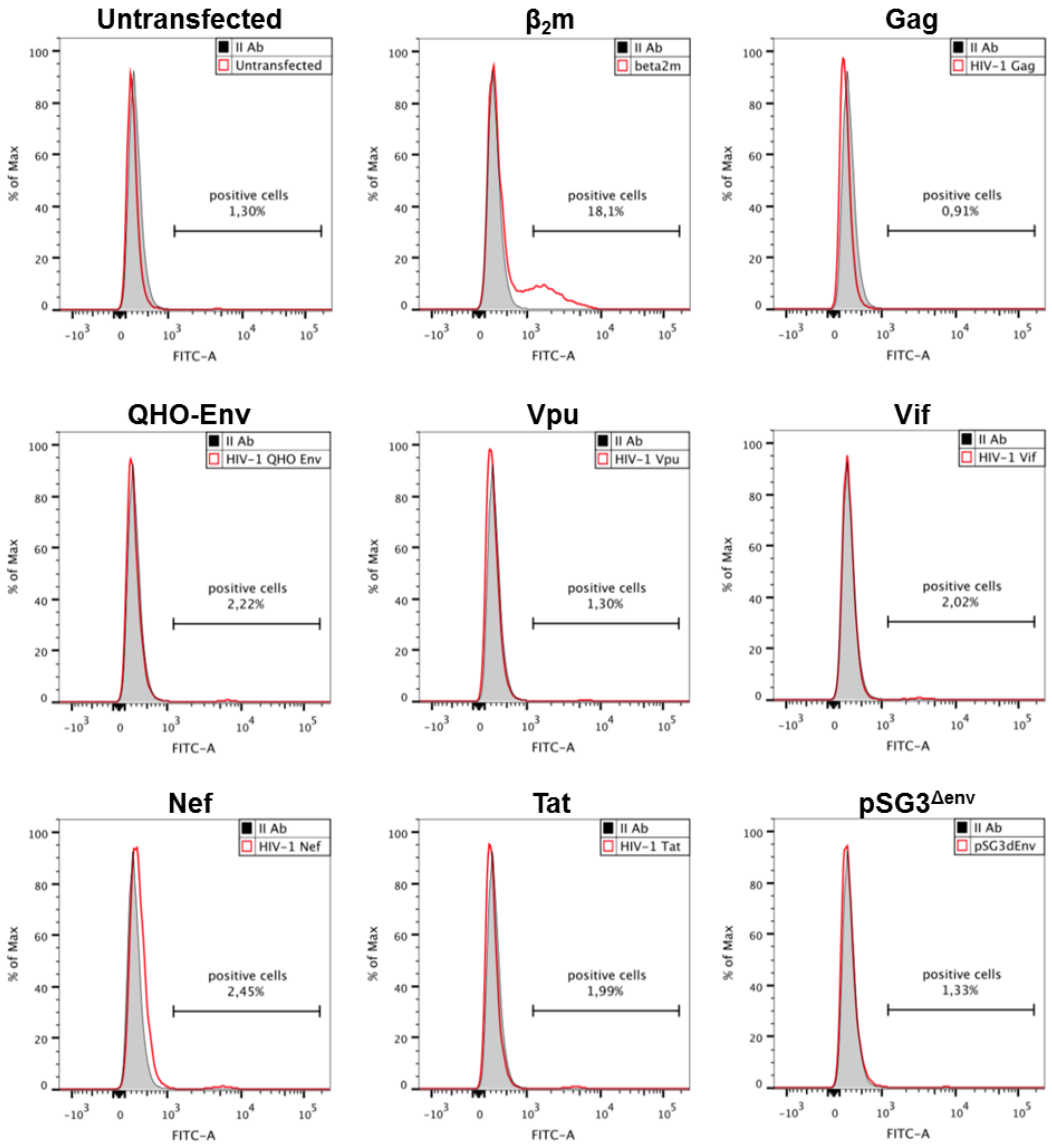


Figure 20. L31 reactivity of β_2m negative HEK-293T cells transfected with different HIV-1 proteins.
Shaded grey curve: Secondary antibody control; red line: L31 reactivity. After transfection with different viral proteins, cells were surface labelled using L31 antibody, after acid wash. HEK-293T cells transfected with the β_2m encoding plasmid were used as control. Cytometry analyses indicate that only β_2m transfection is able to restore HLA-C surface expression (Serena, Parolini et al. 2017).

β_2m knockout variants of both HeLa and HeLa-Lai cells were prepared using the CRISPR/Cas9 system. The HLA-C free chains surface expression was tested by flow cytometry in both parental and β_2m negative cells, after acid wash. Both β_2m negative cell lines were negative for the HLA-C surface expression, suggesting that HIV-1 Env in HeLa-Lai cells couldn't restore the HLA-C surface expression and that β_2m is strictly required for the MHC-I assembly and transport to the cell membrane (Figure 21) (Serena, Parolini et al. 2017).

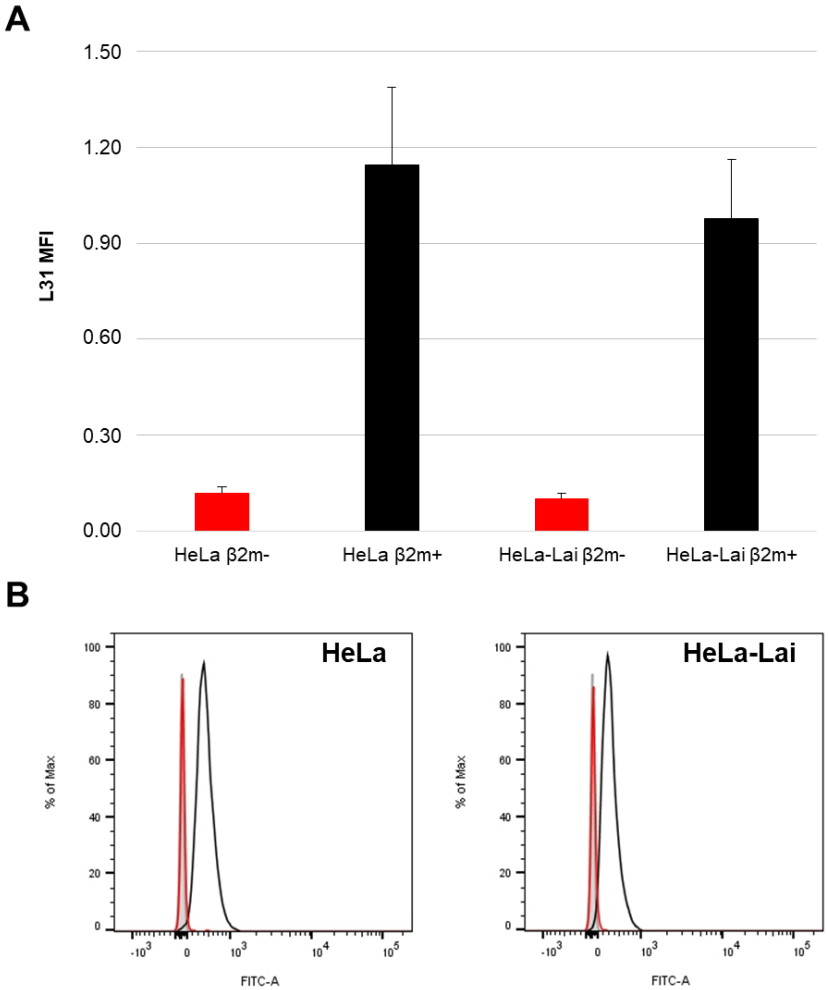


Figure 21. Flow cytometry analyses of HLA-C free chains at the cell membrane in β_2m positive and negative HeLa and HeLa-Lai cells.
A. Histogram graph represents the L31 MFI of HeLa and HeLa-Lai β_2m negative (red) and positive (black) cells. Experiment was repeat for 4 times. Standard errors are reported.
B. Shaded grey curve: Secondary antibody control; red line: L31 surface reactivity of β_2m negative cells; black line: L31 surface reactivity of β_2m positive cells. Only the parental cells showed HLA-C surface expression. Adapted from: (Serena, Parolini et al. 2017).

HIV-1 Env/HLA-C association

To study the association between Env and HLA-C at the cell surface, HIV-1 Env expressing HeLa-Lai cells were used to co-purify HLA-C with Env. Thus, HeLa-Lai cells were treated with the cell membrane insoluble, thiol-cleavable DTSSP reagent (Lomant and Fairbanks 1976) to crosslink protein complexes on the cell surface. Proteins lysates were then purified on a *Galanthus nivalis* lectin column that binds D-mannose groups bound to the HIV-1 Env protein. After

column wash, several elution fractions were collected at increasing concentrations of methyl α-D-mannopyranoside. Purified complexes heavier than 100 KDa were reduced and analysed by western blot (Figure 22A). As negative control, identical amounts of proteins from control HeLa cells were analysed following the same protocol. To exclude any excessive cross-linking of membrane proteins due to DTSSP treatment, Flotillin-1 (a membrane protein) expression was tested as control: no specific co-purification in the presence of HIV-1 Env was observed, and no differences in its purification were revealed between HeLa and HeLa-Lai cells. In HeLa lysates, HLA-C molecules were eluted in the first fractions due to the low content of mannoses (Ryan and Cobb 2012), whereas HLA-C molecules produced in HeLa-Lai cells were co-purified, in parallel with Env, in high amounts and in all the eluted fractions. Furthermore, co-immunoprecipitation of complexes from the cell surface of both Hela and HeLa-Lai cells with the anti Env (2G12) antibody confirmed the HIV-1Env/HLA-C association (Figure 22B). Analysis of the HLA-C heterotrimeric complexes (DT9 antibody) did not result in HIV-1 Env co-immunoprecipitation, excluding a role of HLA-C heterotrimers in the association with HIV-1 Env protein. These results suggest that HIV-1 Env associates with HLA-C in its free chains conformation at the cell membrane.

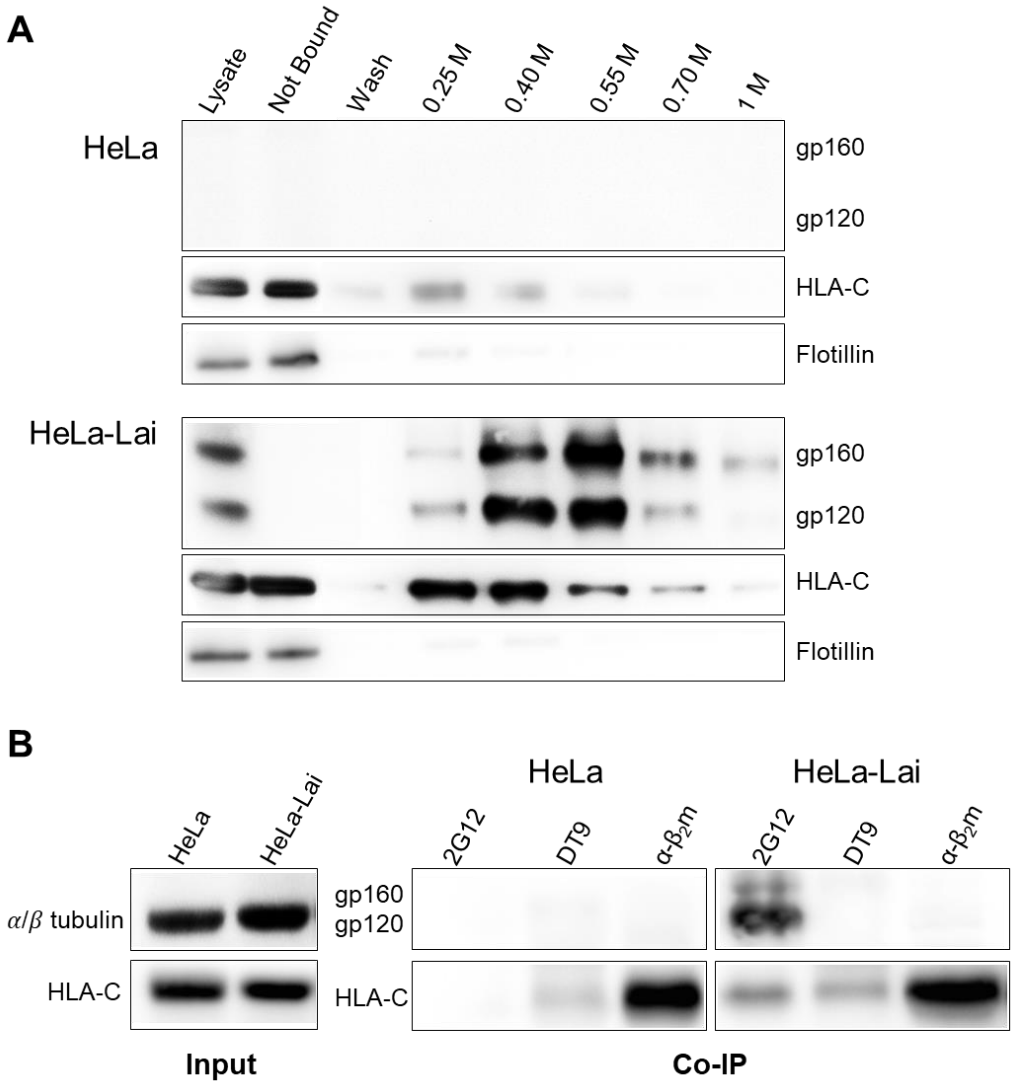


Figure 22. HIV-1 Env and HLA-C are associated at the cell surface.
A. GN lectin columns of protein extracts from HeLa and HeLa-Lai cells, after surface treatment with the cross linker DTSSP. Protein expressions of Env, HLA-C and flottilin-1 was tested by western blot on purified complexes heavier than 100 KDa. As expected, Env purification is present in HeLa-Lai cells but not in HeLa cells. HLA-C molecules, containing few mannoses, are eluted in the first fractions from HeLa cells, while they are co-purified in higher amounts from HeLa-Lai cells, in the presence of HIV-1 Env. No differences in flottilin-1 purification were detected.
B. Membrane complexes from HeLa and HeLa-Lai cells were immunoprecipitated using different antibodies: 2G12 (anti HIV-1 Env), DT9 (anti HLA-C heterotrimeric complexes) and α-β_2m (anti β_2m) antibodies. Western blot analyses of immunoprecipitated samples show that HLA-C is associated either with β_2m or with HIV-1 Env. α/β tubulin expression was detected as a control (Serena, Parolini et al. 2017).

β_2m role in modulating HIV-1 infectivity

Previous studies showed that HIV-1 Env specifically associates with HLA-C, improving the ability of HIV-1 virions to infect host cells (Cosma, Blanc et al. 1999, Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010). To investigate the role of β_2m in virion infectivity, Env-pseudotyped viruses (QHO and pRHPA) HIV-1 isolates were produced in HEK-293T positive and negative β_2m cells. An infectivity assay was performed using TZM-bl cells. The TZM-bl cell line is stably transfected with a plasmid encoding for the luciferase regulated by the HIV-1 Tat promoter. Exploiting this cellular system is possible to quantify the viral infectivity measuring the luciferase expression signal. For the experiment, equivalent amounts of pseudoviruses, determined by titration of p24, were used. The viral infectivity of pseudoviruses produced in HEK-293T β_2m positive cells was significantly higher than the one of pseudoviruses produced in HEK-293T β_2m negative cells (Figure 23, two-way ANOVA, $p < 0.0001$). By analysing the interpolating curve, best fitting the obtained Relative Luminescence Units (RLU) values, an infectivity increase 3 fold higher for pseudovirueses produced by β_2m positive cells compared to the infectivity of pseudoviruses produced in β_2m negative cells was observed. No difference was observed when the protein G of the unrelated pseudovirus Vesicular Stomatitis Virus (VSV) was used. These data suggest that the β_2m can increase HIV-1 infectivity ensuring the HLA-C transport at the cell membrane, where it associates with Env.

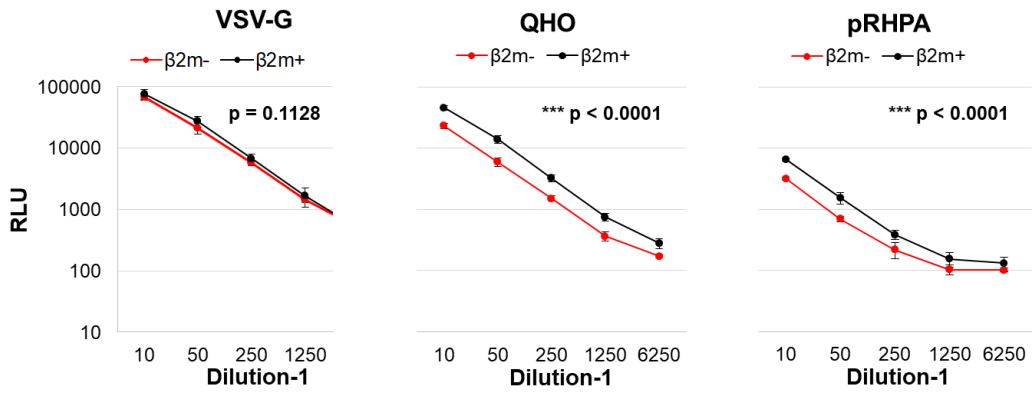


Figure 23. Infectivity assay on TZM-bl cells of HIV-1 psuedoviruses produced in β_2m positive/negative HEK-293T.
Pseudoviruses produced in HEK-293T β_2m positive cells (black line) show a significantly higher infectivity than those produced in HEK-293T β_2m negative cells (red line). This observation is true for two HIV-1 Env pseudotyped viruses, called QHO and pRHPA and not for the unrelated pseudotyped virus VSV-G. Infectivity is expressed as Relative Luminescence Units (RLU). The technical quadruplicates are reported and the error bars represent standard deviations. p values refer to two-way ANOVA analyses, comparing β_2m positive and negative pseudoviruses. Adapted from: (Serena, Parolini et al. 2017).

PBMC donor population analysis

PBMC were collected from healthy bone marrow donors afferent to the Italian Bone Marrow Donor Register (IBMDR) and followed by the Service of Transfusional Medicine (AOUI) in Verona. They were typed for HLA-A, -B and -C by the high resolution molecular biology methods (Reverse PCR-SSO and Luminex Technology). The ethic Committee in Verona, approved this study on 14 October 2015 (ProgCE678CESC). All the samples were collected after written informed consent was obtained from the donors.

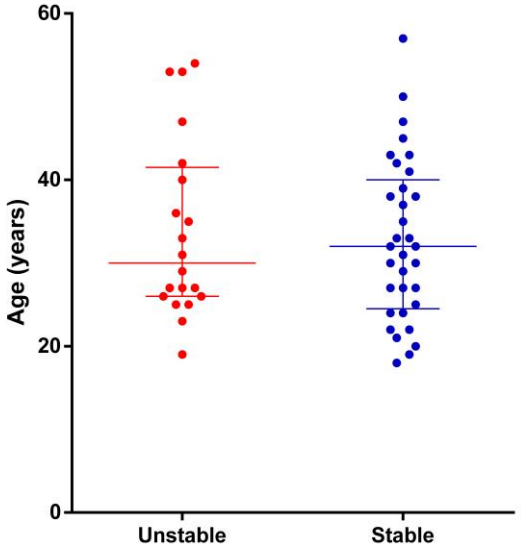
Since different HLA-C allotypes might present different binding stability to β_2m/peptide and may display different expression levels, subjects with one Unstable and one Stable HLA-C allele were excluded because they probably show intermediate phenotypes. To emphasises differences between the two groups, we selected donors having both HLA-C alleles belonging either to the Unstable or to the Stable group. In addition, donors harboring cross-reactive HLA-B alleles (B*13:01, *35:01, *40:06 and *73:01 for the DT9 antibody and B*07, *08, *22, *35, *46, *51, *54 and *56 for the L31 antibody) were not included. Thus, only about 10% of potential donors was suitable for this study (Table 1).

Donor	Sex	Age	HLA-A	HLA-B	HLA-C	Group
1	M	19	*02:01, *30:01	*40:01, *58:01	*03:02, *03:04	U
2	M	37	*11, *68	*37, *44	*06, *16	S
3	M	31	*02, *29	*18, *58	*07, *07	U
4	M	35	*02, *33	*14, *44	*05, *08	S
5	M	29	*02, *24	*44, *58	*07, *07	U
6	F	20	*02:01, *03:01	*27:05, *44:02	*02:02, *05:01	S
7	M	36	*02, *26	*15, *55	*03, *03	U
8	M	18	*11:01, *29:02	*39:01, *44:03	*12:03, *16:01	S
9	F	21	*25:01, *68:02	*39:01, *45:01	*12:03, *16:01	S
10	F	26	*02, *02	*15, *15	*04, *04	U
11	M	41	*24:02, *25:01	*18:01, *44:03	*12:03, *16:01	S
12	M	24	*02, *02	*44, *49	*05, *15	S
13	M	27	*02:01, *24:02	*38:01, *44:02	*05:01, *12:03	S
14	M	25	*02:01, *11:01	*38:01, *44:03	*12:03, *16:01	S
15	M	47	*02:01, *02:01	*44:05, *44:05	*02:02, *02:02	S
16	M	27	*02:01, *26:01	*15:01, *49:01	*04:01, *07:01	U
17	M	25	*23, *32	*15, *44	*01, *04	U
18	M	25	*02:01, *03:01	*15:01, *18:01	*03:03, *07:01	U
19	F	33	*26, *03	*13, *44	*05, *06	S
20	M	29	*11:01, *30:04	*44:02, *50:01	*06:02, *16:04	S
21	F	22	*02, *33	*14, *39	*08, *12	S
22	M	42	*03, *24	*39, *44	*04, *04	U
23	M	38	*02:01, *26:01	*18:01, *38:01	*12:03, *12:03	S
24	F	23	*11, *23	*18, *44	*04, *07	U
25	M	38	*26, *26	*37, *37	*06, *06	S
26	M	31	*01:01, *26:01	*38:01, *57:01	*06:02, *12:03	S
27	F	30	*02:01, *03:01	*13:02, *50:01	*06:02, *06:02	S
28	M	42	*02:01, *30:02	*18:01, *49:01	*05:01, *06:02	S
29	M	47	*31:01, *03	*18:01, *40:01	*03:04, *07:01	U
30	M	30	*30:02, *68:01	*18:01, *44:02	*02:02, *05:01	S
31	M	19	*01:01, *32:01	*14:01, *57:01	*06:02, *08:02	S
32	M	27	*02:01, *26:01	*18:01, *58:01	*07:01, *07:01	U
33	M	32	*33:01, *34:02	*14:02, *14:02	*08:02, *08:02	S
34	M	26	*03:01, *29:02	*39:06, *58:01	*07:01, *07:02	U
35	F	24	*01:01, *01:01	*37:01, *57:01	*06:02, *06:02	S
36	M	27	*01:01, *32:01	*41:02, *49:01	*07:01, *07:03	U
37	F	33	*01:01, *24:03	*18:01, *18:01	*07:01, *07:01	U
38	M	22	*02:01, *03:01	*38:01, *44:02	*05:01, *12:03	S
39	M	32	*01:01, *26:01	*37:01, *38:01	*06:02, *12:03	S
40	M	43	*11:01, *25:01	*18:01, *52:01	*12:02, *12:03	S
41	F	27	*25:01, *33:01	*14:02, *44:02	*05:01, *08:02	S
42	F	27	*03:01, *25:01	*13:02, *18:01	*06:02, *12:03	S
43	M	35	*02:01, *02:01	*18:01, *44:02	*07:01, *07:04	U
44	M	33	*03:01, *11:01	*14:01, *18:01	*05:01, *08:02	S
45	F	53	*24:02, *32:01	*37, *44	*01:02, *04:10	U
46	F	54	*11:01, *23:01	*44:02, *38:02	*04:01, *07:02	U
47	M	53	N.A.	*49, *58	*07, *07	U
48	M	40	N.A.	*15, *49	*07, *07	U
49	M	50	*02:01, *29:01	*18, *44	*08:09, *16:01	S
50	M	39	*24:02, *30:11	*13:02, *37:01	*06, *06	S
51	M	43	N.A.	*37, *44	*06, *16	S
52	M	57	N.A.	*38, *52	*12, *12	S
53	M	45	*01:01, *02:05	*15:03, *49:01	*06, *12	S

N.A.: not available

Table 1. Summary of study population.
The table reports the Sex (M, male; F, female), MHC-I typing (HLA-A, -B, -C) and HLA-C stability group (S, Stable; U, Unstable). Adapted from: (Parolini, Biswas et al. 2017).

The two donor groups analysed in this study were homogeneous and did not show any difference neither for gender, (χ^2 = 0.950), nor for age (p = 0.6919) (Figure 24).



	Unstable	Stable
Age (years)	33.9 ± 2.41	32.76 ± 1.69
Gender male %	75.00	75.76

Figure 24. Study population.
The dot graph represents the age distributions of the two analysed populations (red: Unstable group, blue: Stable group). The bars represent the median and quartiles for each group. No significant difference was detected (t-test).
The table summarizes the age and the sex distributions in the two analysed populations.

Comparing the frequencies of HLA-C alleles of IBMDR donors with the frequencies of HLA-C alleles reported in Northern Italy (Guerini, Fusco et al. 2008), some differences were observed. Specifically an increase of HLA-C*05, C*06 and C*08 and a decrease of HLA-C*04 frequencies were observed. By comparing the frequencies of HLA-C alleles of the selected donors population with the frequencies of HLA-C alleles of the IBMDR donors we observed an increase of HLA-C*05, C*08 and C*12 frequencies and a decrease of HLA-C*04 and C*15 frequencies in the selected population (Figure 25). These differences are likely due to the exclusion from the present study of donors with cross-reactive HLA-B alleles which are in linkage disequilibrium with specific HLA-C alleles,

since haplotypes tend to be inherited in block. In fact, some common allotypes in the Italian population are reported to be B*35:01-C*04:01 and B*51:01-C*15:02 (Allele Frequency Net Database AFND; http://www.allelefrequencies.net) (Gonzalez-Galarza, Takeshita et al. 2015).

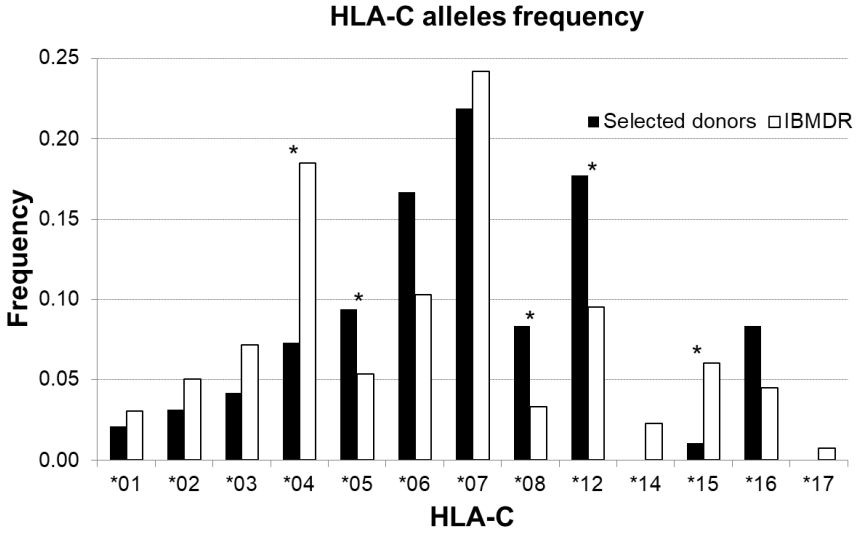


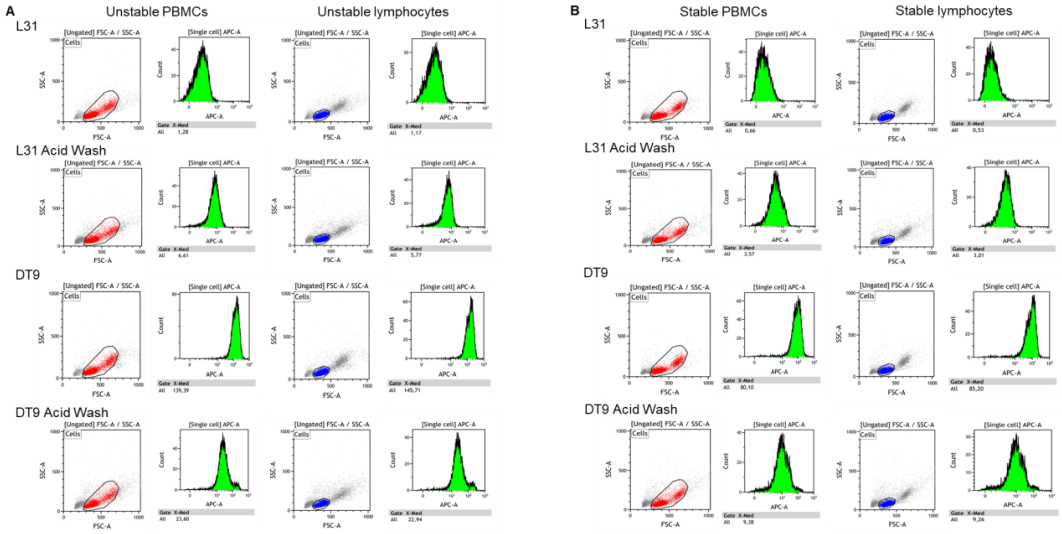
Figure 25. HLA-C frequencies of selected donors and IBMDR afferent donors. Histograms represent the HLA-C frequencies distribution in IBMDR afferent donor (white bars) and selected donors (black bars). The stars indicate the significant differences between the two distributions, due to the stringent selection operated (Fisher's exact test).

Different HLA-C conformational expression on PBMC surface

Preliminary experiments revealed the absence of differences in HLA-C surface stability between lymphocytes and whole PBMC population. An illustrative comparison between PBMC and lymphocytes of donors harboring Unstable (Figure 26A) and Stable (Figure 26B) HLA-C alleles is reported. Thus, all the following analyses were performed on PBMC.

The HLA-C surface expression on selected donors PBMC was assessed using different antibodies. Flow cytometry analysis performed using the DT9 antibody (which recognizes the heterotrimeric complex HLA-C/β_2m/peptide) revealed no significant difference between the two groups (Figure 27A, Wilcoxon test, p = 0. 6712).

When the analysis was conducted after acid wash treatment (which detaches β_2m from HLA-C) a significantly lower DT9 reactivity was observed in the Unstable group compared to the Stable one (Figure 27B, Wilcoxon test, p = 0.0147), underlining the presence of a small pool of stable HLA-C trimers on PBMC harboring Unstable HLA-C variants. As expected, the DT9 fluorescence expressed as Relative Median Intensity (RMFI) after acid wash was lower than the RMFI of the constitutive DT9 (Figure 27A and 27B), reflecting the disruption of the majority of HLA-C heterotrimeric complexes.



		PBMCs	Lymphocytes
Unstable	Fold change L31	4.26	4.10
	Fold change DT9	8.16	8.53

		PBMCs	Lymphocytes
Stable	Fold change L31	7.91	8.52
	Fold change DT9	7.64	7.74

Figure 26. **Gating strategies and illustrative histograms for the flow cytometry experiments**. DT9 mAb recognizes heterotrimers; L31 mAb recognizes HLA-C free chains; Acid Wash, enables the β_2m/peptide removal from HLA-C heterotrimers. A donor harboring both Unstable (A) and a donor with both Stable (B) HLA-C alleles are showed. Fold changes were calculated for mAb L31, as the ratio between RMFI after and prior to acid wash, and for mAb DT9, as the ratio between RMFI prior to and after acid wash. RMFI was calculated as reported in Materials and Methods section. $MFI_{control}$ (secondary antibody control) was 0.19 or 0.26 for Unstable PBMC, 0.15 or 0.20 for Unstable lymphocytes, 0.17 or 0.15 for Stable PBMC and 0.12 or 0.10 for Stable lymphocytes, respectively without or with previous acid wash. Fold change analyses conducted on PBMC and on lymphocytes of the same donor show similar results for both antibodies, as showed in the tables (Parolini, Biswas et al. 2017).

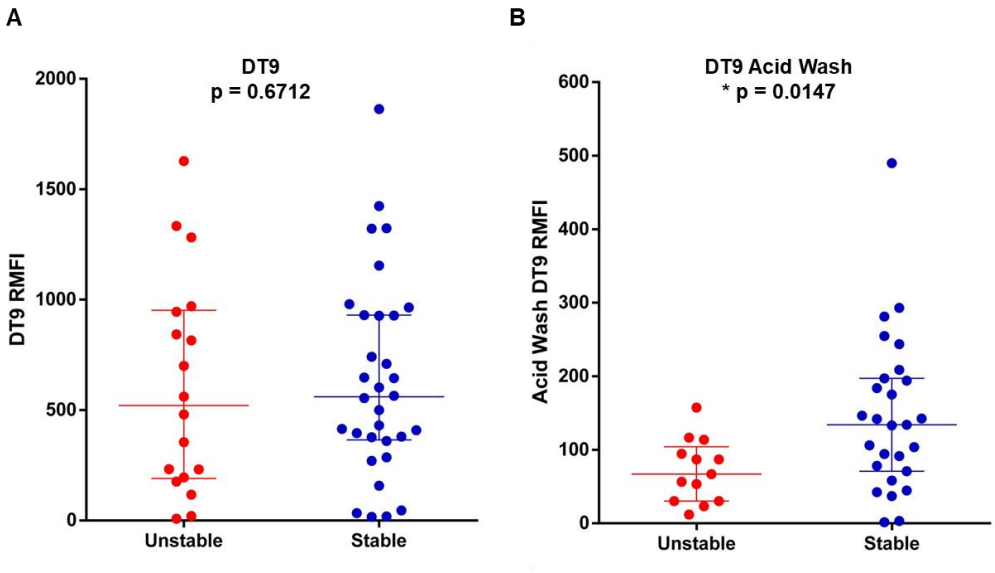


Figure 27. Flow cytometry analysis of DT9 reactivity before and after acid wash. Flow cytometry analyses of HLA-C/β_2m/peptide trimeric complexes detected using the DT9 antibody, on PBMC surface, before (A, Unstable n=18, Stable n=32) and after (B, Unstable n=13, Stable n=27) acid wash. The bars represent the median and quartiles of each group. No significant differences between the two analysed populations were observed (Wilcoxon-Mann-Whitney test). Adapted from: (Parolini, Biswas et al. 2017).

Surface staining with the L31 mAb displayed similar levels of HLA-C free chains between the two analysed groups (Figure 28A, Wilcoxon test, p = 0.9415). Only a slight difference was detected when the L31 analysis was performed after acid wash, which permits the quantification of a larger fraction of HLA-C free chains expressed on the PBMC surface of the Stable group (Figure 28B, Wilcoxon test, p = 0.0563). As expected, the L31 RMFI values prior to acid wash were lower than the RMFI of the L31 RMFI after acid wash, as visible by the different scale (Figure 28A and 28B).

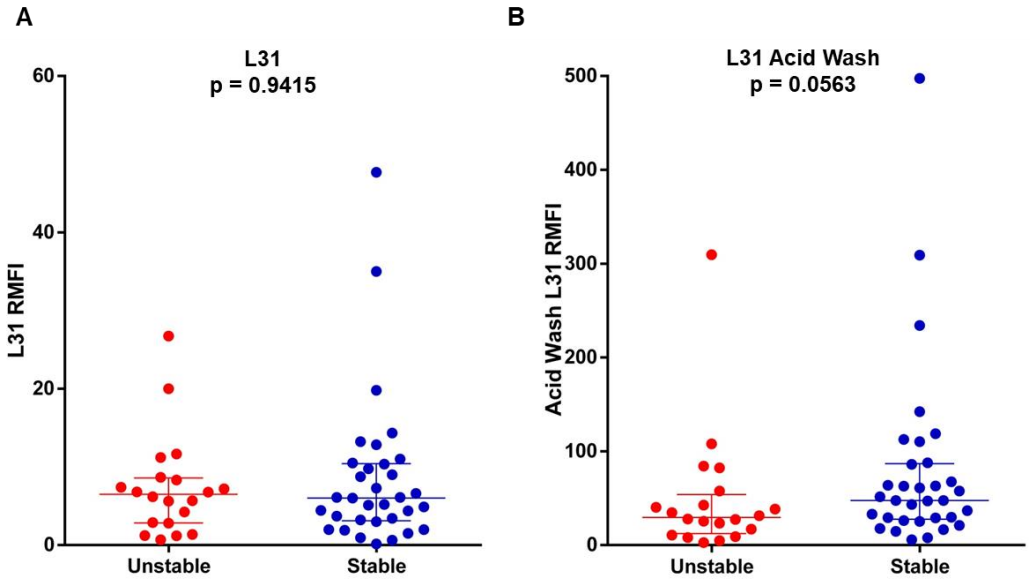


Figure 28. Flow cytometry analysis of L31 reactivity before and after acid wash. Flow cytometry analysis of HLA-C free chains performed using the L31 antibody, on PBMC surface, before (A, Unstable n=20, Stable n=33) and after (B, Unstable n=20, Stable n=33) acid wash treatment. The bars represent the median and quartiles of each group. No significant differences between the two analysed populations were observed (Wilcoxon-Mann-Whitney test). Adapted from: (Parolini, Biswas et al. 2017).

Fluorescence fold change in PBMC

The HLA-C/β_2m/peptide binding stability was evaluated by calculating the fluorescence fold change as the ratio between L31 RMFI after and prior to the acid wash treatment, to determine the amount of HLA-C free chains molecules originally present on PBMC surface and released after the β_2m stripping. If the fraction of HLA-C in its free chains conformation is higher compared to HLA-C heterotrimers, this ratio will be slightly influenced by the acid wash and the L31 fluorescence fold change will be moderately low. *Vice-versa*, if HLA-C molecules are mostly present as heterotrimeric complex, the acid wash treatment will have a greater effect in switching the HLA-C conformation and thus the L31 fold change ratio will be higher. The working hypothesis is that HLA-C allotypes classified as Unstable are less stably associated to β_2m/peptide and consequently present a higher proportion of free chains than Stable allotypes. Therefore, a lower and higher fluorescence fold change increase is expected for the Unstable and Stable HLA-C allotypes, respectively.

Indeed, flow cytometry analyses showed that PBMC from donors belonging to the Stable group have a significant higher fluorescence fold change compared to those belonging to the Unstable group (Figure 29A, Wilcoxon test, p = 0.0063). We did not note the prevalence of some specific HLA-C allotypes in those subjects having the highest fluorescence fold change value, excluding any involvement of specific HLA-C variants. On the contrary, the DT9 RMFI fold change was comparable between the two analysed groups (Figure 29B, Wilcoxon test, p = 0.1794).

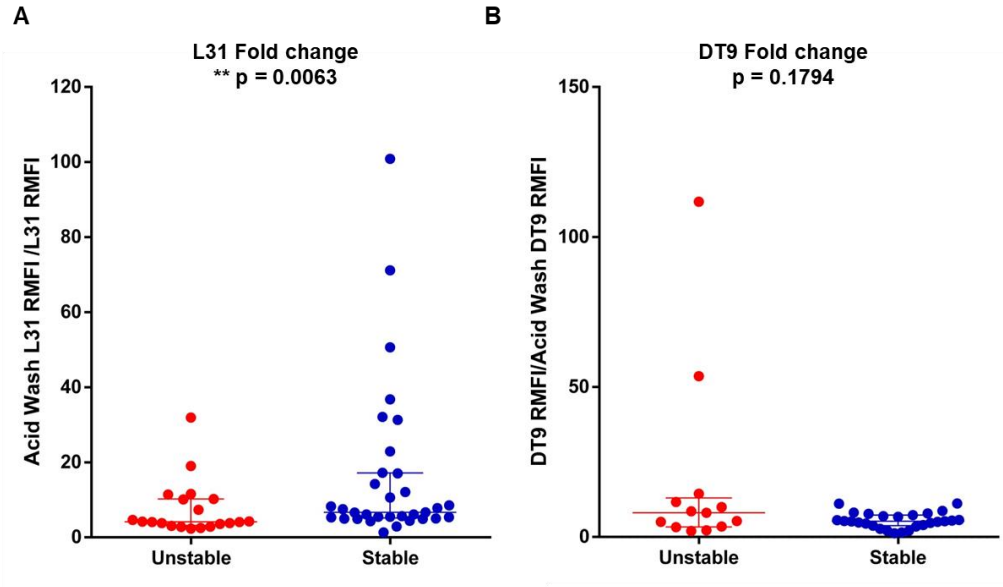


Figure 29. L31 and DT9 fluorescence fold change.
A. L31 fluorescence fold change, calculated as the ratio between L31 RMFI after and before acid wash. This value is statistically significant higher for the Stable group, compared to the Unstable one (Wilcoxon-Mann-Whitney test). Unstable n=13, Stable n=27.
B. DT9 fluorescence fold change, calculated as the ratio between DT9 RMFI before and after acid wash. This value is not statistically significant different in the two groups. The bars represent the median and quartiles of each group. Unstable n=13, Stable n=27. Adapted from: (Parolini, Biswas et al. 2017).

HLA-C and β_2m total expression in PBMC

To test the total HLA-C and β_2m expression levels, western blot analyses were performed on PBMC lysates. No significant difference in the expression levels of β_2m was detected (Figure 30A, Wilcoxon test, p = 0.2109). *Vice-versa*, a significantly higher total expression of HLA-C (Figure 30B, Wilcoxon test, p =

0.0312) was observed in the Stable group. The significantly higher total HLA-C expression observed in the Stable group, could be due to the higher expression levels of some HLA-C variants (i.e., *02, *06, *15), associated with SNP rs67384697 (Kulkarni, Savan et al. 2011) and SNP rs2395471 (Vince, Li et al. 2016), grouped as Stable, and the lower expression of other variants (i.e. *03, *07) classified in the Unstable group.

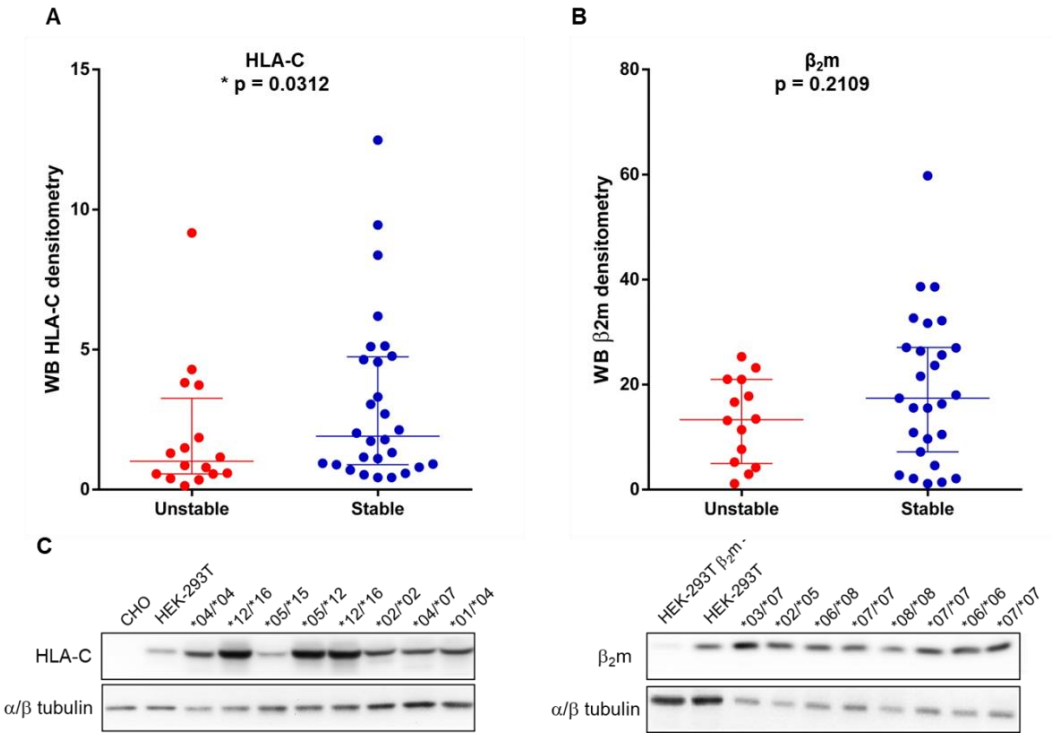


Figure 30. HLA-C and β_2m total expressions in PBMC.
A. Total HLA-C expression assessed by western blotting using L31 antibody. The Stable group present a significant higher HLA-C expression than the Unstable one (Wilcoxon-Mann-Whitney test). The bars represent the median and quartiles of each group. Unstable n=16, Stable n=28.
B. Total β_2m expression assessed by western blotting. No difference was observed between the two groups (Wilcoxon-Mann-Whitney test). The bars represent the median and quartiles of each group. Unstable n=14, Stable n=27.
C. Representative images of western blot experiments used to quantify HLA-C (left) and β_2m (right) expression levels. CHO and HEK-293T cell lysates are negative and positive controls for HLA-C quantification, and HEK-293T β_2m negative and HEK-293T cell lysates for β_2m quantification, respectively. For the quantification method refer to the Material and Methods section. The HLA-C allotypes are indicated for the reported PBMC samples. Adapted from: (Parolini, Biswas et al. 2017).

HLA-C allotypes modulate HIV-1 infectivity

Infection assay of PBMC is dependent on several different elements (such as, number of $CD4^+$ and $CD8^+$ T lymphocytes, expression level of HIV-1 co-receptors, PHA activation, cell growth, cell viability), thus a standardized system to compare the HLA-C alleles influence on HIV-1 infectivity was set up. PBMC from a donor belonging to the Unstable group and a donor belonging to the Stable one, were randomly coupled and tested the same day, in the same experimental set. PBMC were infected with two HIV-1 strains, a prototype of an R5-tropic variant and a prototype of an X4-tropic one. After p24 titration, the same amount of virus produced was used to infect TZM-bl target cells (Platt, Wehrly et al. 1998). PBMC from 16 donors (8 with Unstable and 8 with Stable HLA-C alleles) were infected with HIV-1 BaL (R5-tropic strain) and IIIB (X4-tropic strain). A significant lower infectivity (Figure 31A, three-way ANOVA, $p < 0.0001$) was observed in TZM-bl cells infected with the R5-tropic BaL HIV-1 isolate propagated in PBMC with Stable compared to PBMC with Unstable HLA-C alleles. On the contrary, no significant difference was observed using the X4-tropic HIV-1 IIIB isolate (Figure 31B, three-way ANOVA, $p = 0.5557$).

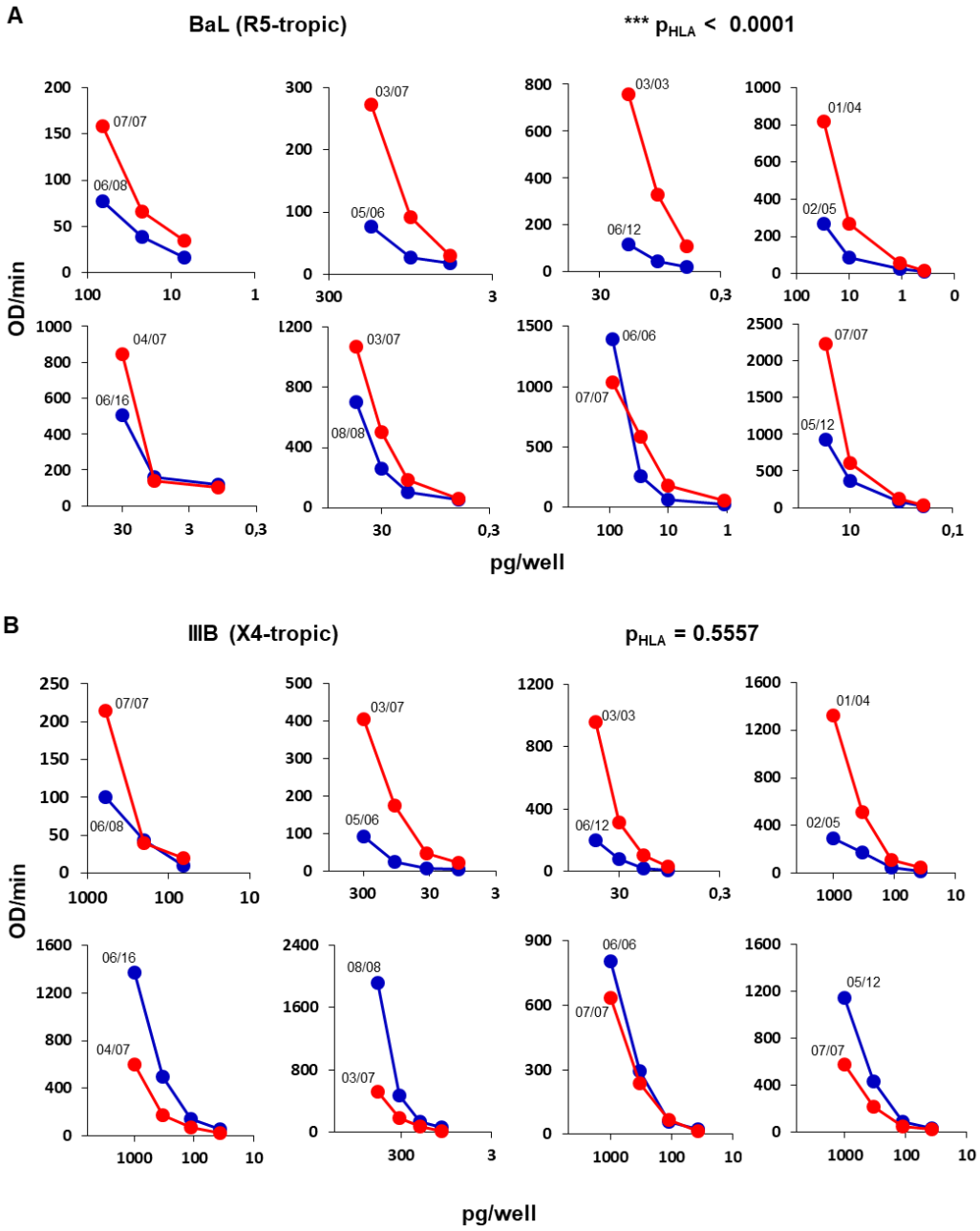


Figure 31. HIV-1 infectivity of virions produced by PBMC. Graphs represent HIV-1 infectivity of different randomly coupled subjects harboring Stable (blue) and Unstable (red) HLA-C alleles. Y-axis refers to OD/min values, which reflect the X-Gal staining signals. The p value for the global analysis (p_{HLA}) is determined by three-way ANOVA and is referred to differences between Stable and Unstable HLA-C alleles.
A. Infection conducted with the BaL HIV-1 isolate.
B. Infection conducted with the IIIB HIV-1 isolate.

To simplify the analysis, for each experimental set, the first viral concentration not showing an evident cytopathic effect on cell viability was used. Data were analysed by two-way ANOVA, untangling the effect of the variability between experimental sets and the effect of HLA-C stability on viral infectivity. A

significant lower infectivity with the R5-tropic BaL HIV-1 isolate was observed for the Stable group compared to the Unstable one (Figure 32A, left panel, $p_{(HLA)} < 0.0001$). No significant difference was observed with the X4-tropic IIIB HIV-1 isolate (Figure 32B, left panel, $p_{(HLA)} = 0.6785$). As expected, significant differences, due to the experimental set, were observed in both cases ($p_{(EXP)} < 0.0001$ and $p_{(EXP)} = 0.0003$ for BaL and IIIB, respectively).

The two groups were finally analysed by comparing the distributions of the mean infectivity for each PBMC sample. The Wilcoxon test showed a significant difference between the two groups for BaL (Figure 32A, right panel, p = 0.0357), but not for IIIB infectivity (Figure 32B, right panel, p = 0.5995).

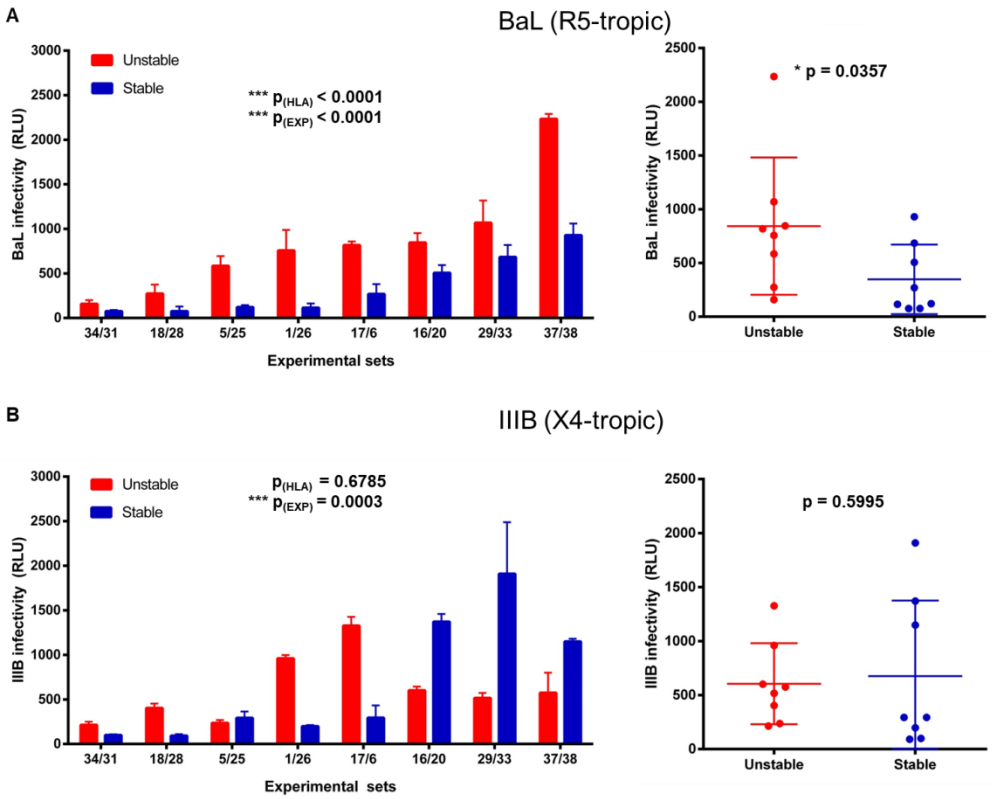


Figure 32. HIV-1 infectivity in donors PBMC.
Histogram charts represent means and standard deviations of HIV-1 infectivity of the different randomly coupled donors harboring both Stable (blue) and Unstable (red) HLA-C alleles. X-axis, experimental set; Y-axis, virions infectivity expressed as RLU. Statistical differences were evaluated by two-way ANOVA: $p_{(HLA)}$ refers to virions infectivity due to the HLA-C group (Stable/Unstable), while $p_{(EXP)}$ refers to the expected experimental set variability.
Dot charts represent the distributions of the mean infectivity of each subject (Unstable HLA-C variants, red dots; Stable HLA-C variants, blue dots). Horizontal bars indicate median and quartiles; data were statistically analysed by Wilcoxon-Mann-Whitney test.
A. Infection conducted with the R5 BaL HIV-1 strain.
B. Infection conducted with the X4 IIIB HIV-1 strain. Adapted from: (Parolini, Biswas et al. 2017).

Fluorescence fold change analysis and HIV-1 infectivity in a controlled cellular model

The differences observed in PBMC were further confirmed on 721.221-CD4 cells. These cells, bearing a deletion of the MHC-I locus, were transfected in parallel with HLA-C*07 or HLA-C*06 expressing plasmids, respectively an Unstable and a Stable HLA-C allele. Flow cytometry analyses of 4 independent transfection experiments showed a significantly higher L31 fluorescence fold change for the HLA-C*06 expressing cells compared to the HLA-C*07 ones, after β_2m removal from the cell surface (Figure 33A, two-way ANOVA, $p = 0.0297$). This result confirms the observation previously made on PBMC. The experiment was performed on a controlled cellular model, thus excluding any other variable due to the immune system or individual differences between PBMC donors, further supporting the working hypothesis.

Finally, infectivity of HIV-1 virions produced by 721.221-CD4 cells transfected either with HLA-C*07 or HLA-C*06 was evaluated. Because of 721.221-CD4 cells express only the CXCR4 HIV-1 co-receptors, they were used to assess the IIIB HIV-1 infectivity in the presence of the HLA-C*07 (Unstable) or -C*06 (Stable) allele. The supernatants were quantified for their p24 content and then the same virus amounts were used to infect TZM-bl cells. Infectivity of virions produced by 721.221-CD4-C*06 cells was significantly lower than that of virions produced by 721.221-CD4-C*07 cells (Figure 33B, two-way ANOVA, $p = 0.0001$).

This experiment confirms, in a cellular model, that HIV-1 has a lower infectivity when produced in the presence of Stable HLA-C alleles.

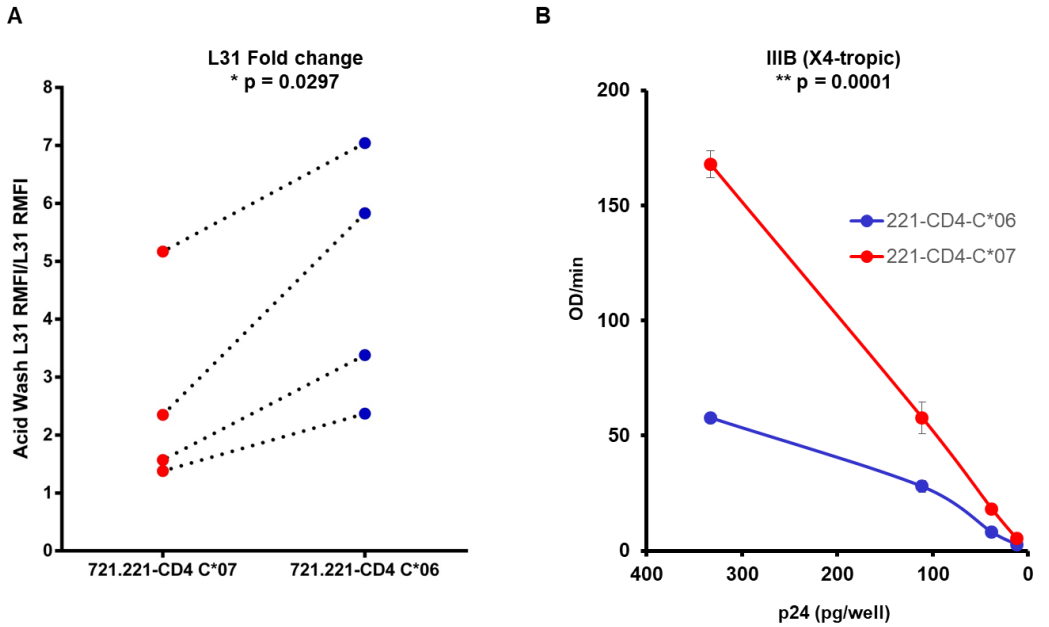


Figure 33. HLA-C allotypes stability and their effect on HIV-1 infectivity.
A. Fluorescence fold change analyses in 721.221-CD4 cells. L31 RMFI fluorescence fold change in HLA-C*07 (Unstable, red dots) and HLA-C*06 (Stable, blue dots) expressing 721.221-CD4 cells. The graph displays the flow cytometry results of 4 independent transfections. The same transfection experiment is connected by the dotted line. Two-way ANOVA was applied for the statistical analysis.
B. Infectivity of HIV-1 IIIB virions produced by 221-CD4-C*06 (Stable variant) and 221-CD4-C*07 (Unstable variant) cells. Four different p24 concentrations were used to infect TZM-bl target cells in triplicate cultures (standard deviations are reported). X axis: p24 pg/well; Y-axis: virions infectivity expressed as OD/min. Red: virions produced in 721.221-CD4-C*07 cells; blue: virions produced in 721.221-CD4-C*06 cells. Adapted from: (Parolini, Biswas et al. 2017).

HLA-C alleles differences in binding stability and expression levels

It could be speculated that the outcome of HIV-1 infection might depend not only on the amount of HLA-C expressed on the cell surface, but also on its stability as trimeric complex. According to this model, subjects with low expressed HLA-C alleles and unstable binding to β_2m/peptide present less immunocompetent HLA-C heterotrimeric complexes, and more HLA-C free chains available for the interaction with HIV-1 Env. They might thus have a worse immunologic control of HIV-1 infection, as well as an intrinsically lower ability to counteract viral replication. On the contrary, individuals with highly expressed HLA-C alleles and stable binding stability of HLA-C/β_2m/peptide present a higher proportion of HLA-C immunocompetent heterotrimeric complexes and less HLA-C free chains.

Therefore, these subjects are expected to have a better immunologic control of HIV-1 infection and produce a less infectious virus (Figure 34).

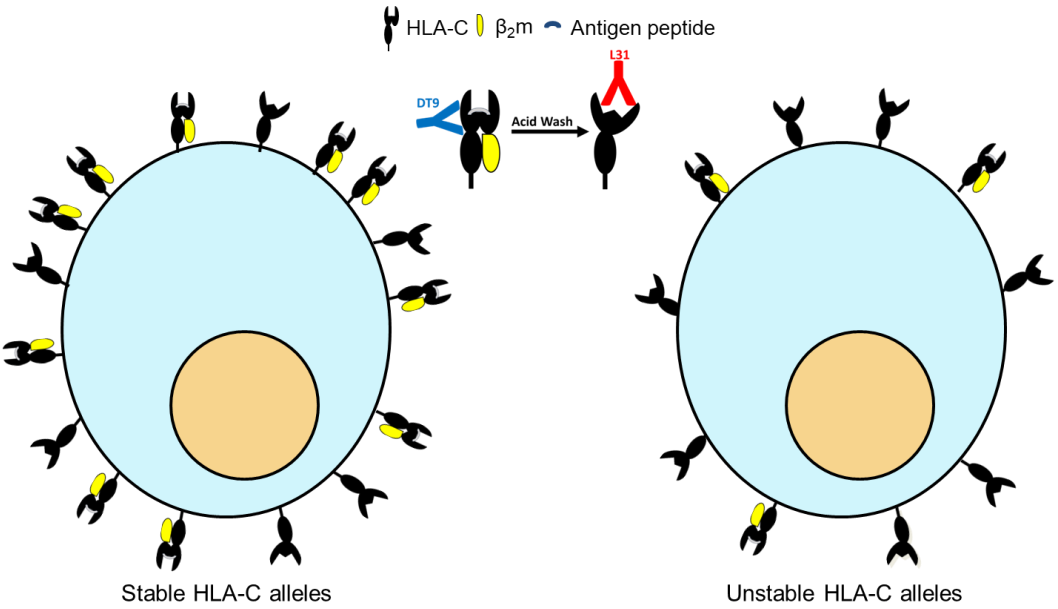


Figure 34. Expression and stability of HLA-C alleles on the cell membrane. DT9 mAb (light blue) recognizes HLA-C/β_2m/peptide complexes, while L31 mAb (red) recognizes HLA-C free chains. PBMC of subjects harboring Stable HLA-C alleles express proportionally more heterotrimers and less free chains on the cell membrane (left), while PBMC of subjects with Unstable HLA-C alleles express proportionally less heterotrimers and more HLA-C free chains.

Role of exogenous β_2m on HIV-1 infectivity

To test if HIV-1 Env originates HLA-C free chains because the virus needs the β_2m to modulate its infectivity, different concentrations of soluble β_2m, during the TZM-bl infectivity assay were tested.

Exogenous soluble β_2m did not influence the HIV-1 QHO-Env pseudotyped virions infectivity, at the two used dilutions (Figure 35A, QHO 1:250, $p = 0.2119$; QHO 1:500, $p = 0.0534$). This result suggests that soluble β_2m is not able to interfere with the infectivity of HIV-1 virions lacking the β_2m on their surface. Probably β_2m lacking virions could not take it from the medium.

Finally, the effect of β_2m on HIV-1 infectivity was assessed testing if the presence of an anti β_2m antibody during virions production affects infectivity, hampering or favoring the β_2m release from virions. HIV-1 Env pseudotyped viruses were

produced in the presence of the anti-β_2m antibody (BBM1) and in the presence of an unrelated antibody. Virions produced in the absence of any antibodies were used as control (Figure 35B). No significant differences were observed, neither with the BBM1 antibody (p = 0.5952) nor with the unrelated anti PTPRG antibody (p = 0.9606). This result suggests that the HIV-1 virions exploit the HLA-C free chains to increase their infectivity, and not the β_2m.

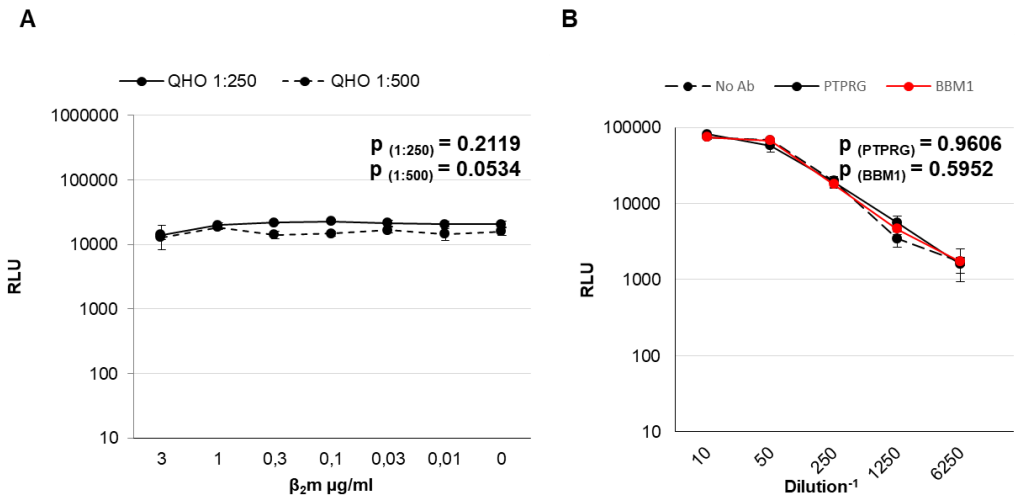


Figure 35. Infectivity assay on TZM-bl cells of HIV-1 pseudoviruses.
A. HIV-1 pseudoviruses negative for β_2m were used to infect TZM-bl target cells in the presence of different β_2m concentrations. Two dilutions of pseudoviruses produced in HEK-293T β_2m negative cells (solid line 1:250, dashed line 1:500) were used to infect TZM-bl target cells in the presence of different concentrations of β_2m (from 0 µg/ml to 3 µg/ml). Y-axis: infectivity expressed as Relative Luminescence Units (RLU). X-axis: β_2m concentrations. The experiment was performed in triplicate, the error bars represent standard deviations. Two-way ANOVA was used to ascertain statistical significant differences.
B. HIV-1 Pseudoviruses expressing β_2m were produced in the presence of the BBM1, anti β_2m, antibody (solid red line). As controls pseudoviruses were produced in the absence of any antibody (dashed black line) and in the presence of an unrelated antibody, PTPRG (solid black line). Y-axis: infectivity, expressed as Relative Luminescence Units (RLU). X-axis: viral dilutions. The experiment was performed in triplicate, the error bars represent standard deviations. Three-way ANOVA was used to ascertain statistical significant differences.

Time course analysis of HLA-C/ β_2m/peptide dissociation rate

To test the strength of the HLA-C/β_2m/peptide stability, A3.01 cells were treated with acid wash for different times and then surface labelled with the L31 mAb. After cytofluorimetric analyses, L31 fluorescence fold changes were calculated. The maximum β_2m detachment occurred after 2 minutes of acid wash (Figure 36). At later times a remarkable L31 reactivity decrease was observed, probably due to HLA-C free chains disruption. Further experiments will be performed focusing in the range 0-2 minutes of acid wash treatment.

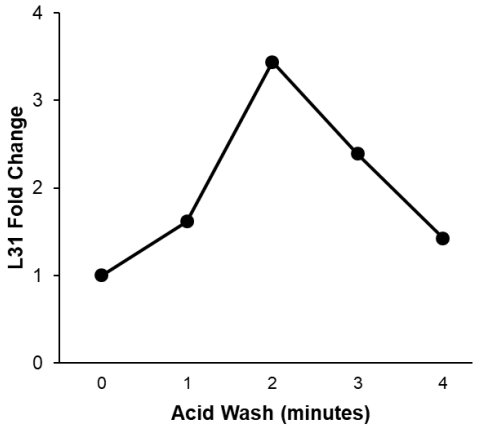


Figure 36. HLA-C/β_2m/peptide binding stability in A3.01 cells. A3.01 cells treated with acid wash for different times were surface stained with the L31 antibody and analysed at cytofluorimeter. Graph represents the L31 fluorescence fold changes at different times.

Discussion

It is known that HIV-1 virions incorporate several host cell proteins during the budding process, among which HLA-C (Esser, Bess et al. 2001). Several studies reported the interaction between HIV-1 Env and HLA-C and it is known that HLA-C expression levels contribute to the control of HIV-1 infection. High HLA-C expression levels have been associated with a better lymphocyte activation and a slow progression towards AIDS (Apps, Qi et al. 2013), while low HLA-C expression levels have been related to a rapid progression to the disease. HLA-C molecules have been found associated with Env on the viral envelope and in the absence of HLA-C both fusion efficiency and viral infectivity are reduced (Cosma, Blanc et al. 1999, Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010). In this work, a significant up regulation of surface-HLA-C free chains (L31 antibody reactivity) (Setini, Beretta et al. 1996) was observed in HIV-1 infected TNF alfa stimulated cells (ACH-2 cells). The same HLA-C free chains surface increase was not detected in the corresponding stimulated uninfected parental cell line (A3.01 cells). Noteworthy, no difference in HLA-C heterotrimers surface expression (DT9 antibody reactivity) (Braud, Allan et al. 1998) between the HIV-1 infected and uninfected cells was observed, suggesting that HIV-1 reactivation selectively induces HLA-C free chains appearance at the cell membrane. Moreover, a time course experiment revealed that the HLA-C free chains expression at the cell surface correlated with the expression of HIV-1 Env: they both decrease upon 48 and 72 hours of TNF-α stimulation. Interestingly, no differences in the cell membrane expression of total HLA-C molecules, HLA-C heterotrimers, MHC-I or β_2m were observed at any time, suggesting that HIV-1 selectively raises the amount of surface expressed HLA-C free chains. The same phenomenon was observed in another cellular model (PM1 cells), where HIV-1 reactivation induced by TNF-α stimulation correlated with the increase of HLA-C free chains at the cell surface. Moreover, HEK-293T cells transfected with a HIV-1 Env (QHO isolate) expressing plasmid presented a higher HLA-C free chains surface expression than HEK-293T cells transfected with the Env defective

full length HIV-1 genome plasmid (pSG3$^{\Delta env}$), indicating that the presence of the Env protein could induce the appearance of HLA-C free chains at the cell membrane.

HIV-1 infection does not directly induce the HLA-C increase, neither at the expression level nor by modulating its membrane translocation. Thus, it is likely that the presence of HIV-1 Env facilitates the dissociation of β_2m from HLA-C, generating HLA-C free chains. β_2m plays a crucial role in the HLA-C translocation at the cell surface. None of the HIV-1 tested proteins are able to restore the HLA-C surface expression in the absence of β_2m. HLA-C needs the β_2m light chain to reach the plasma membrane, and once there, HLA-C could dissociate from β_2m in the presence of HIV-1 Env protein. Experiment conducted using the BiFC technique (Serena, Parolini et al. 2017), revealed a very close association between Env and HLA-C free chains at the cell membrane. The HIV-1 Env/HLA-C free chain association was confirmed both by proteins purification on GN lectin column and by co-immunoprecipitation of membrane protein complexes, on HeLa-Lai cells.

HLA-C molecules are characterized by an inefficient assembly with the peptide, which in turn leads to the accumulation in the endoplasmic reticulum of both β_2m-associated and β_2m-free folding intermediates (Sibilio, Martayan et al. 2008). This could explain why HLA-C expression level is lower compared to those of HLA-A and -B. It is known that MHC-I free chains, due to their long half-life, can cis-associate both with themselves and with other membrane receptors, such as CD3, CD8αβ, CD25, Ly49A and IL-15Rα (Tremblay, Fortin et al. 1998, Esser, Bess et al. 2001, Arosa, Santos et al. 2007, Ott 2008). Thus, it is likely that HLA-C free chains might be able to associate with Env. Since β_2m is essential for the proper assembly of MHC-I molecules and their translocation to the cell membrane, the role of β_2m in HIV-1 infectivity was explored. In particular, an infectivity assay on TZM-bl target cells was performed, revealing that the infectivity of HIV-1 pseudoviruses produced in β_2m negative HEK-293T cells was roughly 3 times lower than the infectivity of pseudoviruses produced in the β_2m positive HEK-293T cells. A similar 3-fold reduction in HIV-1 infectivity was also observed by Cosma *et al*. in the absence of HLA-C (Cosma, Blanc et al.

1999). This data is supported by previous experiments showing that silencing of HLA-C expression negatively affects HIV-1 infectivity (Matucci, Rossolillo et al. 2008). This result further confirms the HLA-C involvement in modulating HIV-1 infectivity (Matucci, Rossolillo et al. 2008).

However, since β_2m knockout in HEK-293T cells also suppresses the expression of other classical and non-classical HLA-Class I molecules, the observed reduction in viral infectivity could not be exclusively due to the absence of HLA-C on the cell surface. In addition, virions lacking β_2m on their surface did not recover their infectivity in the presence of soluble exogenous β_2m, suggesting that HIV-1 Env does not require β_2m to modulate viral infectivity.

HIV-1 Env binds the HLA-C in its free chains conformation and the release of β_2m might be a side effect. It is not yet clear if HIV-1 Env is able to actively induce the HLA-C heterotrimer dissociation displacing the β_2m, or if β_2m spontaneously dissociate from the heterotrimer generating HLA-C free chains available for the interaction with HIV-1 Env. Overall, the reported results, together with other previously published data (Cosma, Blanc et al. 1999, Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010), demonstrate that HLA-C interacts with HIV-1 Env and in particular that the increased infectivity conferred to the virus by HLA-C is due to HLA-C free chains. HLA-C may increase HIV-1 infectivity in different ways: HLA-C could assist the virion assembly, or it could stabilize the Env proteins through a cis-interaction, reducing the shedding of viral glycoproteins from the cell surface and/or from the viral particle. Moreover, it is known that HLA-C reduces HIV-1 virions susceptibility to neutralizing antibodies (Cosma, Blanc et al. 1999).

Once discovered that HLA-C free chains are crucial in influencing HIV-1 infectivity, it was investigated on PBMC from healthy donors if different HLA-C variants present different binding stability to β_2m/peptide and whether the relative proportions of HLA-C free chains and HLA-C heterotrimeric complexes could be involved in modulating HIV-1 infectivity. In order to address this aspect, it was necessary to set a boundary between the groups. As in many biological phenomena, there is no “black” and “white”, but different degrees of stability may be expected. Thus, different HLA-C alleles were classified in two different

groups (Stable/Unstable), depending on the bond stability between HLA-C and β_2m/peptide according to Sibilio *et al* (Sibilio, Martayan et al. 2008). The working hypothesis predicts a lower proportion of HLA-C free chains on PBMC isolated from donors with Stable HLA-C alleles compared to PBMC obtained from individuals with Unstable HLA-C variants.

In the present work HLA-C expression on PBMC surface was evaluated using two different antibodies specific for the HLA-C β_2m-free and β_2m-associated (both constitutive and after β_2m/peptide removal) to avoid the underestimation of the real amounts of HLA-C molecules exposed on the cell surface (Serena, Parolini et al. 2017), as reported in previous studies (Corrah, Goonetilleke et al. 2011, Apps, Qi et al. 2013) evaluating only the HLA-C heterotrimeric complexes expression. The strength of this study, although performed on a small population, derives from the stringent selection carried out, which includes only donors having both HLA-C alleles belonging to the Unstable or Stable group and excludes any HLA-B allotypes cross-reactive with the L31 and DT9 used mAbs. At the same time, the selection exemplifies an extreme situation to validate the testing hypothesis. Certainly, in a general population, a continuous range of susceptibility degrees to HIV-1 infection is expected.

Both HLA-C heterotrimeric complexes (DT9 reactivity) and HLA-C free chains (L31 reactivity) showed comparable levels in the Stable and Unstable considered group, indicating that the total amount of HLA-C expressed at the cell surface, although highly variable, is comparable in the two groups. Differences previously reported (Apps, Qi et al. 2013) were not observed. This is probably due to the necessary exclusion of subjects carrying L31 and DT9 cross-reactive HLA-B alleles. Moreover, the different classification between Unstable and Stable HLA-C alleles, could explain why differences previously reported by others were not observed. The same cytofluorimetric analyses was carried out upon acid wash treatment. It is, however, worth pointing out that after acid wash the DT9 and L31 reactivities have undergone significant decrease and increase, respectively.

Noteworthy, after acid wash treatment, a slightly higher L31 and DT9 reactivities were observed in the presence of Stable HLA-C alleles, suggesting that, although the global amount of HLA-C present at the steady state on the cell surface was

similar in the two groups, differences do exist in the proportion of the HLA-C heterotrimeric complexes and HLA-C free chains.

Moreover, the relative amount of HLA-C β_2m-free and β_2m-associated was evaluated by calculating the L31 fluorescence fold change, which reflects the HLA-C/β_2m/peptide binding stability. Hence, this value was significantly higher in the presence of HLA-C Stable compared to Unstable variants. The same result was confirmed in the 721.221-CD4 cellular model, where HLA-C*06 or HLA-C*07 substitute the deletion of the MHC-I locus. HLA-C*07 (an Unstable HLA-C allele) expressing cells presented a lower L31 fold change compared to HLA-C*06 (a Stable HLA-C allele) expressing ones. This observation supports previous data, and in addition excludes any other genetic or immunological factors, which may interfere in the experiments performed on PBMC. No significant differences were observed in the DT9 fluorescence fold change

The significant difference in the L31 fluorescence fold change confirms the working hypothesis: which is that Stable and Unstable allotypes present a stronger and a weaker stability as trimeric complexes, respectively. Hence, it is possible to define as “Unstable” the HLA-C variants *01, *03, *04, *07 and possibly *14, and “Stable” the HLA-C variants *02, *05, *06, *08, *12, *15 and *16. Thus, the HLA-C expression level on the cell surface is the result of the combination of two distinct important phenomena: the level of the HLA-C expression and the peptide-affinity and stability of the HLA-C/β_2m/peptide heterotrimeric complexes. Adding complexity, the expression level depends both on HLA-C mRNA regulation mediated by miRNA 148a (Thomas, Apps et al. 2009, Apps, Qi et al. 2013) and by the SNP rs2395471 in the HLA-C promoter region (Vince, Li et al. 2016).

It is important to underline that for some HLA-C variants an overlapping between expression level and heterotrimeric stability does exist. Some low expressed HLA-C variants (Apps, Qi et al. 2013, Vince, Li et al. 2016) such as HLA-C*03 and *07, also have the highest dissociation rate according to Sibilio *et al*. (Sibilio, Martayan et al. 2008), while some Stable HLA-C variants such as C*02, *06, *12 and *16 are also expressed at high levels. As a consequence, the total HLA-C

amount evaluated by western blot, was significantly higher in the Stable group compared to the Unstable one.

On the contrary, this correspondence is not true for HLA-C*01 and *04 alleles, which was defined as highly expressed by Vince *et al*. (Vince, Li et al. 2016), but was reported to have a lower binding stability to β_2m/peptide according to Sibilio *et al*. (Sibilio, Martayan et al. 2008). No HLA-C mRNA expression for these alleles was reported by Vince *et al*., while other groups (Corrah, Goonetilleke et al. 2011, Gentle, Paximadis et al. 2013, Bettens, Buhler et al. 2016) did not show any particular increase in their mRNA expression level. Moreover, HLA-C*01 and *04 were reported to have a high surface reactivity to mAb DT9 (Apps, Qi et al. 2013), but since they also present a weak bond to β_2m/peptide, it could be possible that the DT9 staining underestimates the real amounts of HLA-C molecules expressed on the cell surface, making the HLA-C surface expression the result of many variables and factors. Thus, the expression of HLA-C heterotrimeric complexes on the cell surface depends on the synergy of several factors such as mRNA levels, post-translational regulation, transport and stability of HLA-C alleles.

Once ascertain that HLA-C could be present in two different conformations on the cell surface, it was tested if the proportion of HLA-C heterotrimers and HLA-C free chains might be important in modulating HIV-1 infection. Results obtained from this work showed that HIV-1 is able to specifically increase the amount of HLA-C free chains in chronically or acutely infected cell lines (Serena, Parolini et al. 2017). Thus, was tested if the infectivity of HIV-1 virions is affected by HLA-C stability. Indeed, the infectivity of HIV-1 BaL R5-tropic virions produced in PBMC of donors having Stable HLA-C alleles was significantly lower than the infectivity of virions produced by PBMC of individuals bearing Unstable HLA-C alleles. According to this finding, virions budding from PBMC carrying Stable HLA-C alleles could be less infectious because a lower proportion of HLA-C free chains would be available to interact with HIV-1 Env (Serena, Parolini et al. 2017), reflecting a reduced infectivity as well as a higher susceptibility to neutralizing antibodies (Cosma, Blanc et al. 1999). On the contrary, HIV-1 IIIB X4-tropic virions appeared to be less dependent on the presence of different

HLA-C alleles, but HIV-1 IIIB virions produced by 721.221-CD4-C*06 were less infectious than those propagated in 721.221-CD4-C*07 cells.

This experiment performed on the same cellular background is noteworthy and confirms the working hypothesis on HIV-1 infectivity and HLA-C stability. The fact that HIV-1 IIIB virions produced by PBMC with Unstable HLA-C alleles were not significantly more infectious than those produced by PBMC with Stable alleles may depend on several variables that occur in PBMC among donors.

Moreover, the R5-tropic variants, which presented a lower infectivity in the presence of Stable HLA-C alleles, are also those that predominate in the first asymptomatic period of infection (Grivel, Shattock et al. 2011), that is precisely the phase for which was reported a correlation between HLA-C alleles and differences of viral load (Fellay, Ge et al. 2009).

In a previous study (Matucci, Rossolillo et al. 2008) it was reported that the infectivity of the CXCR4-tropic isolates, J500 and NDK, was not influenced by HLA-C absence, indicating a reduced effect of HLA-C on these X4-tropic isolates. It could be possible that the effect of Unstable HLA-C variants on the X4-tropic isolates infectivity could be overshadowed by other factors in the PBMC model, while it is apparent in the 721.221-CD4 cellular model, in which the only difference is due to the expression of the Stable (C*06) or Unstable (C*07) HLA-C variant.

The relationship between HIV-1 and HLA-C is a complex interaction, since HIV-1 needs the expression of HLA-C on the cell surface to increase its infectivity (Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010, Zipeto and Beretta 2012), but at the same time, HLA-C can stimulate an appropriate CTL immune response against the virus and thus induce a better infection control (Ward, Bonaparte et al. 2004, Apps, Qi et al. 2013). The demonstration, *in vitro*, that HLA-C variants can differently affect HIV-1 infectivity indicates that the availability of HLA-C free chains is important in modulating viral infectivity without any influence by the host immune system. Indeed, the interaction and combination between different stability as surface trimers, different HLA-C expression levels and of many other host genetic variants can influence the viral set point and the outcome of the infection.

This study, highlighting an important role for different HLA-C conformations in modulating HIV-1 infectivity, explains the apparent contradiction resulted by the previous observations suggesting a controversial role of HLA-C in HIV-1 infection. Indeed, both a protective role of HLA-C against HIV-1 infection, and a role for HLA-C virions incorporation in increasing viral infectivity, were reported. It is possible that the alternative conformations of HLA-C, resulted from the different stability as heterotrimers, could directly affect the balance between protection and susceptibility outcome. It is likely that individuals with Unstable HLA-C allotypes (C*01, *03, *04, *07, *14) are characterized by a larger pool of HLA-C free chains on the cell surface, which in turn can bind HIV-1 Env and increase viral infectivity. On the contrary, subjects with Stable HLA-C allotypes (C*02, *05, *06, *08, *12, *15, *16) are characterized by a higher proportion of HLA-C heterotrimers on the cell surface, which can properly stimulate cellular immunity, and at the same time can present fewer HLA-C free chains available for the interaction with HIV-1 Env.

Of course, variants of other HLA molecules play a crucial role in HIV-infection, but since HLA-A and –B are known to strongly bind β_2m/peptide, most likely they may influence HIV-1 infection outcome by improving the immune response rather than through a direct association with HIV-1 Env. Anyway, HLA-B*46, for instance, which has been reported to be related with increased susceptibility to HIV-1 infection (Triantafilou, Triantafilou et al. 1999) displays a less stable bond with β_2m/peptide according to Sibilio *et al*. (Sibilio, Martayan et al. 2008). In the present study, subjects having HLA-B*46 were excluded due to the cross-reactivity with the L31 mAb.

In conclusion, the results of the present study indicate that the stability of HLA-C complexes and HLA-C expression levels are important in modulating HIV-1 infectivity. The combination of these two both equally important phenomena, can help in the prediction of the infection and disease progression.

Moreover, expression levels alone do not explain why HIV-1 exploits HLA-C on the cell surface to be more infectious (Cosma, Blanc et al. 1999, Matucci, Rossolillo et al. 2008, Baroni, Matucci et al. 2010, Serena, Parolini et al. 2017) and do not explain why in HIV-1 positive patients higher β_2m serum and

cerebrospinal fluid concentrations were observed (Folks, Benn et al. 1985, Schwartz, Alizon et al. 1994, Platt, Wehrly et al. 1998, Bremnaes 2009). According to these novel findings subjects with Unstable HLA-C variants may lose β_2m, determining its accumulation.

HLA-C variants which can more easily dissociate from β_2m/peptide, will probably show a higher proportion of free chains available for the HIV-1 Env interaction and, at the same time, they probably present a relevant release of β_2m, which in turn contributes to inflammatory states. It could be interesting to investigate if HIV-1 Env protein helps the spontaneous decay of HLA-C heterotrimeric complexes, as previously reported for CMV (Grundy, McKeating et al. 1987), originating more HLA-C free chains available for the interaction with Env. Indeed, studies published in the early years of HIV-1 pandemic, described that patients with HIV-1 dementia had a higher concentration of β_2m in cerebrospinal fluid (McArthur, Nance-Sproson et al. 1992), that AIDS-Related-Complex (ADC) patients had higher levels of β_2m in serum (Lacey, Forbes et al. 1987) and that these levels raise during HIV-1 infection progression (Sonnerborg, von Stedingk et al. 1989, Hofmann, Wang et al. 1990). The results of the present study start to shed some light on these never entirely clarified observations: subjects with Unstable HLA-C alleles might lose β_2m more easily, leading to its accumulation in the cerebrospinal fluid and in serum. The exact role of β_2m in neurodegeneration is not yet well well-defined, but it is known that β_2m can form fibrils (Yamaguchi, Hasegawa et al. 2001, Yamamoto, Hasegawa et al. 2005) and become neurotoxic (Giorgetti, Raimondi et al. 2009, Smith, He et al. 2015). Furthermore, β_2m has been detected not only on HIV-1 virions (Capobianchi, Fais et al. 1994), but also on HTLV-I (Timar, Nagy et al. 1987), echo viruses (Ward, Bonaparte et al. 2004) and coxsackievirus (Triantafilou, Triantafilou et al. 2000) viral particles. The presence of β_2m on these viruses confers protection from neutralizing antibodies, and modulates their infectivity. Noteworthy, β_2m bound to HIV-1 virions is recognized by a specific antibody, which is able to neutralize different HIV-1 isolates but does not bind to β_2m expressed on the cell surface; hence, this epitope, called R7V, is exposed only when β_2m is bound to HIV-1 particles (Bremnaes 2009). A natural continuum of the present work is the study

aimed to evaluate the frequencies of Stable/Unstable HLA-C alleles in patients affected by Alzheimer's Disease or AIDS Dementia Complex. A higher prevalence of Unstable HLA-C alleles in patients population compared to a control population would be expected, since these Unstable alleles more easily lose the β_2m, leading to the development of neurodegenerative processes.

As future perspectives, it could be of interest to test a wider panel of HIV-1 isolates, to validate the results obtained with BaL and IIIB on PBMC. The limited availability of sample material from PBMC donors restricted the experiments that could be performed. Moreover, future studies could be aimed to investigate whether HIV-1 infection affects levels and distribution of HLA-C heterotrimers/free chains in PBMC, and in $CD4^+$ HIV-1 infected T cells, in relation to SNP rs2395471 and rs67384697.

Besides it could be of interest to deeply investigate the dissociation rate of the HLA-C/β_2m /peptide complex in the presence of different HLA-C variants. For instance homozygous donors for HLA-C alleles could be analysed by time course analysis for their PBMC heterotrimeric complexes stability upon increasing time of acid wash treatment. Preliminary unpublished experiments conducted on A3.01 cells (expressing *03 and *07 unstable HLA-C alleles) revealed, that the maximum dissociation occurs after 2 minutes of acid treatment, and that longer treatment completely destroy the entire complex, breaking down L31 mAb reactivity.

This study, investigated the complicated relationship between HLA-C and HIV-1, focusing on the different HLA-C conformations and their ability to modulate viral infectivity interacting with HIV-1 Env protein. The understanding of this interaction can assist in the design of new therapeutic strategies, such as therapeutic monoclonal antibodies and derivatives, binding the regions of interaction between HIV-1 Env and HLA-C, aimed at controlling HIV-1 infection, as well as to understand the role of HLA-C and β_2m in neuro-inflammatory diseases.

Materials and Methods

Buffer and solutions

Laemmli buffer	50 mM Tris (Sigma-Aldrich) - HCl pH=6.8
	6% v/v glycerol (Euroclone)
	3% v/v β-mercaptoethanol (Sigma-Aldrich)
	1% w/v SDS (Sigma-Aldrich)
	0.001% w/v bromophenol blue (Sigma-Aldrich)
Non-denaturing lysis buffer	150 mM NaCl (Sigma-Aldrich)
	1% v/v Triton X-100 (Sigma-Aldrich)
	Protease inhibitor cocktail tablets (Roche)
RIPA buffer	50 mM Tris (Sigma-Aldrich) - HCl pH=7.4
	150 mM NaCl (Sigma-Aldrich)
	2 mM EDTA (Sigma-Aldrich)
	1 mM PMSF (Thermo Fisher Scientific)
	1% v/v Triton X-100 (Sigma-Aldrich)
	1% v/v Sodium deoxy cholate (Sigma-Aldrich)
	Protease inhibitors cocktail tablets (Roche)

Lysis Buffer for G-lectin column	2.5 mM HEPES 145 mM NaCl 1% Triton 0.1 mM PMSF
Dialysis buffer	2.5 mM HEPES 145 mM NaCl pH 7.4 0.1% Triton
Running buffer	25 mM Tris (Sigma-Aldrich) 192 mM Glycin (Euroclone) 0.1 % w/v SDS (Sigma-Aldrich)
Running gel (8% - 10% - 15%)	8% -10% - 15% v/v acrylamide (Euroclone) 375 mM Tris (Sigma-Aldrich) - pH 8.8 0.1% w/v SDS (Sigma-Aldrich) 0.1% w/v APS (Sigma-Aldrich) 0.06 v/v Temed (Thermo Fisher Scientific)
Stacking gel (5%)	125 mM Tris (Sigma-Aldrich) - pH= 6.8 0.1% w/v SDS (Sigma-Aldrich) 0.1% w/v APS (Sigma-Aldrich)

	0.1% v/v Temed (Thermo Fisher Scientific)
TBS-T	150 mM NaCl (Sigma-Aldrich)
	20 mM Tris (Sigma-Aldrich)
	0.05% v/v Tween-20 (Sigma-Aldrich)
Transfer buffer pH=7.6	25mM Tris (Sigma-Aldrich)
	192 mM Glycin (Euroclone)
	0.1 % w/v SDS (Sigma-Aldrich)
	20% v/v methanol (Sigma-Aldrich)
Acid Wash/Strip solution (pH=2.5)	RPMI-1640 medium (Euroclone)
	drops of HCl 37% (to adjust pH)
RBCLB Lysis Buffer	0.15 M NH_4Cl,
	10 mM $KHCO_3$
	0.1 mM EDTA

Cell lines

Human embryonic kidney (HEK-293T), HeLa and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (ATCC).

HeLa-Lai cells were kindly donated by Dr. Uriel Hazan, Institut Cochin, Paris, France. These cells were obtained by transfection of a HIV-1 provirus in which *gag* and *pol* genes were deleted, while *nef* was replaced by the *dhfr* drug resistance (Schwartz, Alizon et al. 1994).

TZM-bl cells were provided by the EU Programme EVA Centre for AIDS Reagents, NIBSC (ARP5011) (Platt, Wehrly et al. 1998). A3.01 is a $CD4^+$ T-lymphoma cell line, from which is derived the ACH-2 cell line by HIV-1 IIIB latent infection (Folks, Benn et al. 1985). The viral reactivation in ACH-2 cells could be obtained by TNF-α stimulation (Poli, Kinter et al. 1990, Biswas, Smith et al. 1995).

PM1 (from Dr. P. Lusso, AIDS Research and Reference Program, Division of AIDS, NIAID, NIH, USA) is a cellular line derived from the human T lymphocytic HUT 78 cell line. PM1-IIIB are the HIV-1 IIIB chronically-infected counterpart.

721.221-CD4 cells, provided by Prof. A. Siccardi (DIBIT-HSR, Milano, Italy) derive from B-lymphoblastoid cells not expressing HLA-A, HLA-B, or HLA-C due to the disruption of the HLA genetic locus (Shimizu, Geraghty et al. 1988).

HeLa, HeLa-Lai, HEK-293T, TZM-bl and CHO cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose (Euroclone), while A3.01, ACH-2, PM1 and 721.221-CD4 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Euroclone) (Li, Gao et al. 2005). Both the media were supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-Glutamine (Lonza), 100 U Penicillin/ml and 100 U Streptomycin/ml (Lonza). All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2 and were routinely tested for the absence of mycoplasma contaminations.

Vectors

Plasmids encoding for HIV-1 Env, R5 strain pRHPA (ARP2061, Dr. B. H. Hahn and Dr. J. F. Salazar-Gonzalez) (Li, Gao et al. 2005) and R5 strain QHO (ARP2043, Dr. D. Montefiori and Dr. F. Gao) (Li, Gao et al. 2005) were provided by the EU Programme EVA Centre for AIDS Reagents, NIBSC (Courtesy of NIH AIDS Research and Reference Reagent Programme), as well as the pCV1 plasmid, encoding for HIV-1 Tat (ARP2004, Dr. F. Wong-Staal) (Arya, Guo et al. 1985). The pSG3$^{\Delta env}$ backbone plasmid (catalogue number 11051, Dr. J. C. Kappes and Dr. X. Wu) (Wei, Decker et al. 2002), the plasmids encoding HIV-1 Vif (pcDNA-HVif, catalogue number 10077, Dr. S. Bour and Dr. K. Strebel) (Nguyen, llano et al. 2004) and the HIV-1 Gag (catalogue number 11468, Dr. M. D. Resh and G. Pavlakis) (Schwartz, Alizon et al. 1994) were obtained from the NIH AIDS Research and Reference Reagent Program. Plasmids encoding Nef and Vpu were kindly donated by Dr. M. Pizzato (CIBIO, Trento, Italy) (Pizzato, Helander et al. 2007). Finally, the plasmids expressing VSV-G protein (pCMV-VSV-G) and the human β_2m-microglobulin (pBJ1-human β_2m) were purchased from Addgene, as well as the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector. The plasmid pCR-Blunt II-TOPO encoding the β_2m gRNA (crB2M_13) was provided by Dr. C.A. Cowan (Harvard University, MA USA) (Mandal, Ferreira et al. 2014).

Antibodies

The W6/32 antibody (Parham, Barnstable et al. 1979) and the ascitic fluid NAMB-1 anti-β_2m (Pellegrino, Ng et al. 1982) were kind gifts from Dr. P. Giacomini (Regina Elena Hospital, Rome, Italy).

Both the phycoerythrin-conjugated goat anti-mouse and the Alexa Fluor 488 conjugated goat anti-human were purchased from Southern Biotech.

The Alexa Fluor 488-conjugated goat anti-mouse was purchased from Cell Signalling.

The allophycocyanin-conjugated goat anti-mouse was purchased from BioLegend.

All the previous antibodies were used in flow cytometry.

The anti-flotillin-1 was kindly donated by Prof. S. Mariotto.

The anti-β_2m polyclonal rabbit Ab was provided by Abcam.

The anti α/β tubulin rabbit Ab was purchased from Cell signaling.

The anti-mouse IgG HRP Conjugated was purchased from Promega.

The anti-rabbit IgG (Goat) HRP labeled was purchased from Perkin Elmer.

All the previous antibodies were used in western blotting.

The human 2G12 antibody (EVA3064, Dr. D Katinger), was provided by the EU Programme EVA Centre for AIDS Reagents, NIBSC.

The mAb DT9, specific for the HLA-C/β_2m/peptide heterotrimeric complex (Braud, Allan et al. 1998) was kindly donated by Angharad Fenton-May and Persephone Borrow (Nuffield Dept. of Clinical Medicine, University of Oxford, UK).

The mAb L31 (specific for the α1-domain of HLA-C heavy chain, not bound to β_2m) (Setini, Beretta et al. 1996) was kindly provided by Dr. P. Giacomini (Regina Elena Hospital, Rome, Italy).

The previous antibodies were used both in flow cytometry and western blotting.

The anti-β_2m BBM1 antibody was purchased from Abcam, and the anti-PTPRG antibody was kindly donated by Dr. C. Sorio (University of Verona). The last two antibodies were used in the production of Env-pseudotyped viruses.

Transfections

HEK-293T, HeLa and HeLa-Lai cells were transfected with the TransIT-LT1 transfection reagent (Mirus Bio), following manufacturer's instructions. 721.221-CD4 cells were transfected by nucleofection with the VCA-1003 kit (Lonza), using the X-001 program, according to Lonza Nucleofector's instruction. The transfection efficiency for each cell line was evaluated using the pmaxGFP plasmid (3486 bp) provided by Lonza Nucleofector Kit V, and by analysing the GFP expression by cytofluorimetric analysis.

Development of β_2m knockout cell lines using CRISPR/Cas9 system

$2x10^5$ HEK-293T, HeLa, and HeLa-Lai cells were co-transfected with 250 ng of pCR-Blunt II-TOPO β_2m gRNA (Mandal, Ferreira et al. 2014) and 750 ng of pSpCas9 vectors. Antibiotic selection was carried out with 0.5 µg/ml of puromycin for 3 days. After puromycin selection CRISPR/Cas9-treated cells were expanded and tested for the β_2m expression by flow cytometry, using the ascitic fluid NAMB antibody diluted 1:200 in 5% w/v BSA in PBS. The Alexa Fluor 488 conjugated goat anti-human, diluted 1:200 in 5% w/v BSA in PBS was used as secondary antibody. β_2m negative cells were then sorted using the FACSAria II cell sorter (BD Biosciences, San Josè, CA) provided by the Flow Cytometry and Cell Sorting Platform, Center of Applied Research on Cancer–Network (ARC-Net), University of Verona, directed by Dr. Maria Teresa Scupoli (LURM, University of Verona), with the collaboration of Dr. Chiara Cavallini (LURM, University of Verona). A second cell sorting with the W6/32 and APC-conjugated goat anti-mouse antibodies both diluted 1:200 in 5% w/v BSA in PBS was carried out, to achieve a pure β_2m negative population.

A3.01, ACH-2, PM1, HEK-293T and HeLa cells cytofluorimetric analyses

HIV-1 infected cells were handled in the biosafety level 3 (BSL 3) Human Virology Laboratory, directed by Dr. Mauro Malnati, at San Raffaele Hospital, in the Division of Immunology, Transplantation and Infectious Diseases (Milan, Italy), with the collaboration of Dr. Francesca Sironi and Dr. Priscilla Biswas. A3.01, ACH-2 and PM1 cells were cultured either in the absence or in the presence of TNF-α (10 ng/ml) for the different indicated times. Cells were incubated for 30 minutes at 4°C with 2.5 µg/ml of DT9, 1 µg/ml of L31 and W6/32, 5 µg/ml of 2G12 and 1:200 dilution of ascitic fluid for NAMB-1. After PBS washes, all the cells were stained with the secondary antibodies: the phycoerythrin-conjugated anti-mouse and the Alexa Fluor 488 conjugated goat anti-human, diluted 1:200 in 5% w/v BSA in PBS. Cells were fixed with 1%

formaldehyde prior to acquisition with a Gallios flow cytometer (Beckman Coulter). Data were collected and analysed using the FlowJo software (TreeStar, San Carlos, CA). β_2m negative HEK-293T cells (transfected with the different HIV-1 plasmids), as well as parental and β_2m negative HeLa and HeLa-Lai cells, were stained with 1 µg/ml of L31 antibody for 30 minutes at 4°C and then were stained with the secondary antibody: Alexa Fluor 488-conjugated goat anti-mouse, diluted 1:200 in 5% w/v BSA in PBS. Data were acquired with a FACSCanto flow cytometer (BD Bioscience) and analysed using the FlowJo software (TreeStar, San Carlos, CA). For HLA-C free chains analysis (L31 acid wash), cells were treated with RPMI-1640, 20% FBS, pH 2.5 for 3 minutes in ice to remove the β_2m from the heterotrimeric complex before the analysis.

G-lectin column purification

Both HeLa and HeLa-Lai cells were surface-fixed with 1 mM DTSSP (ThermoScientific) according to manufacturer's instructions. Cells were washed with PBS and re-suspended in lysis buffer. Nuclei were pelleted by centrifugation at 1000 xg for 3 minutes and the supernatants were passed over a snowdrop G-lectin column (Galanthus nivalis lectin, Sigma), after equilibration with dialysis buffer. Protein samples were collected in five elution fractions containing increasing concentrations (250 mM, 400 mM, 550 mM, 700 mM and 1 M, one column volume) of methyl α-D-mannopyranoside (Sigma). Each eluted fraction was concentrated using a 100 KDa Amicon Ultra Centrifugal Filter (Millipore). Proteins quantification was performed at spectrophotometer (Eppendorf) using Coomassie Plus Bradford Protein Assay Reagent (ThermoScientific). 25 µg of lysate, and not bound and 1/4 of the total volume of concentrated fractions were incubated for 10 minutes at 98°C in the presence of 3% β-Mercaptoethanol, to disrupt the DTSSP thiol links. All protein samples were separated by SDS-PAGE and analysed by western blot.

Co-immunoprecipitation assay

HeLa and HeLa-Lai cells were labelled at the cell surface with 2G12, DT9, or anti β_2m (Abcam) antibodies, for 45 minutes at 4°C. After PBS wash, cells were re-suspended in 0.5% NP-40 non-denaturing lysis buffer. Cellular nuclei were removed by centrifugation at 1000 xg for 3 minutes. Cell surface labelled proteins complexes were incubated at 4°C with Dynabeads® protein G (Life Technologies) for 45 minutes. Beads were washed and re-suspended in elution buffer containing 2% SDS and 40 mM DL-Dithiothreitol (DTT). After denaturation by boiling at 98°C for 10 minutes, proteins were separated by SDS-PAGE and analysed by western blot.

HeLa and HeLa-Lai western blotting analysis

For the detection of HLA-C, flotillin-1 and α/β-tubulin, proteins were separated on a 10% acrylamide gel. For the detection of HIV-1 Env, proteins were separated on a 8% acrylamide gel. After the SDS-PAGE, proteins were transferred on a PVDF membrane. Immunoblot analyses were performed using the 2G12 for HIV-1 Env detection and the mAb L31 for HLA-C detection (both diluted 1:200 in 5% milk TBS-Tween 0.05%). The anti-flotillin-1 and the anti-α/β-tubulin (diluted 1:500 and 1:2000, respectively, in 5% milk TBS-Tween 0.05%) antibodies were used as controls. Then, cell membranes were incubated with the appropriate secondary antibodies: the HRP-conjugated anti-mouse or the anti-rabbit antibodies (both diluted 1:2000 in 5% milk TBS-Tween 0.05%). Finally, the signal was developed using the ECL Advance™ Western Blotting Detection Kit (Amersham), through the AutoChemi System UVP (BioImaging System).

Production of Env-pseudotyped viruses and TZM-bl assay

Pseudoviruses expressing the *rev/env* sequences of two different HIV-1 subtype-B strains (QHO and pRHPA) were produced (Li, Gao et al. 2005). $1x10^6$ HEK-293T β_2m positive and negative cells were transfected with 1.5 μg of the Env plasmid DNA and 3 μg of the pSG3$^{\Delta env}$ backbone plasmid. 48 hours later, the medium was collected, cellular debris were eliminated by centrifugation and FBS was added to reach 20% final concentration. Pseudoviruses were titrated using the p24 ELISA kit (Aalto Bio Reagents Ltd, Dublin, Ireland) and the same amount of virus was used to infect $1x10^4$ TZM-bl target cells in the presence of 15 μg/ml DEAE-dextran (Li, Gao et al. 2005). After 48 hours from infection luminescence was measured using the Victor^{TM} 3 luminometer (Perkin Elmer) and infectivity was expressed as RLU. The experiment was performed in quadruplicate. VSV-G plasmid was used as a control.

Selection of PBMC donors

The healthy blood donors involved in the study were registered at the IBMDR, afferent to UOC Transfusional Medicine, AOUI Verona, directed by Dr. Giorgio Gandini. All the donors were typed for HLA-A, HLA-B and HLA-C, by medium-high resolution molecular biology techniques. The donors recruitment and typing were planned by Dr. Valentina Muraro under the supervision of Dr. Elisabetta Guizzardi (UOC Transfusional Medicine, AOUI Verona, Italy). Donors were selected to harbor both HLA-C alleles belonged to the Stable (HLA-C*02:02, C*05:01, *C05:15, C*06:02, C*08:02, C*12:03, C*15, C*16:01, C*16:04) or Unstable (HLA-C*01, C*03:02, C*03:04, C*03:03, C*04:01, C*07:01, C*07:04) group. Since the two mAbs used, DT9 and L31, recognize some HLA-B alleles (Giacomini, Beretta et al. 1997, Sibilio, Martayan et al. 2008, Thomas, Apps et al. 2009, Kaur, Gras et al. 2017), subjects expressing DT9 (B*13:01, *35:01, *40:06 and *73:01) and L31 (B*07, *08, *22, *35, *46, *51, *54 and *56) cross-reactive HLA-B allotypes were excluded. According to these criteria, approximately only about 10% of analysed donors were suitable for the

study. This study was approved by ethic Committee of the University of Verona on 14/10/2015 (ProgCE678CESC), and all the samples were collected after written informed consent was obtained.

MHC class I typing

Blood donors samples were collected in vacutainer tubes with EDTA as anticoagulant. Samples were centrifuged at 450 xg for 10 minutes RT, to obtain three fractions: plasma (upper layer), Buffy-Coat (middle-ring enriched of leucocytes and platelets) and erythrocytes (lower layer). Buffy-Coats were processed to extract the genomic DNA using the EZ1 Advanced XL, (Qiagen) and the obtained DNA was quantified using Spectrophotometer (Eppendorf). The amplification was performed by REVERSE PCR-SSO, Luminex technology, using specific primers for the second and the third exons of HLA-A, HLA-B or HLA-C. Protocol was provided by Lagitre. The data analysis was performed using HLA FUSION Software (One Lambda).

PBMC purification

PBMC from suitable donors, were isolated from Buffy-Coat using Ficoll-Paque PLUS (GE-Healthcare). Briefly, concentrated blood was diluted in PBS and stratified on ficoll. After centrifugation at 400 xg for 30 minutes, w/o break/acceleration, PBMC were collected from the ring at the interface between plasma and ficoll layer. After PBS wash, PBMC were treated with Red Blood Cell Lysis Buffer (RBCLB), and then incubated at 37°C for 4 minutes. After PBS wash, PBMC were counted and stored as aliquots in liquid nitrogen and subsequently used for flow cytometric and western blotting analyses, within two weeks, to ensure the reproducibility of the data. Before using, the cells viability commonly exceeded 90% in each sample, as assessed by Tripan Blue dye exclusion. When enough PBMC were available, they were additionally used to test the HIV-1 infectivity.

PBMC cytofluorimetric analyses

PBMC were surface-labelled with either 1 μg/ml L31 or 2.5 μg/μl DT9 mAb for 40 minutes at 4°C, with or without previous acid wash treatment. In order to reach the highest detachment of β_2m and the need to maintain cell integrity and viability, a classical acid wash treatment was used: RPMI-1640, 20% FBS, pH 2.5 for 3 minutes in ice (Luckey, Marto et al. 2001, Lorente, Garcia et al. 2011). After primary antibody incubation, cells were washed with PBS and surface-stained with APC conjugated goat anti-mouse antibody, diluted 1:200 in 5% w/v BSA in PBS. Thus, cells were washed and re-suspended in PBS and analysed with a FACSCanto flow cytometer, provided by Dr. Maria Teresa Scupoli, Head of the Flow Cytometry and Cell Sorting Platform, Center of Applied Research on Cancer-Network (ARC-Net), University of Verona (Verona, Italy). Dead cells and cellular debris were excluded based upon forward scatter (FSC) and side scatter (SSC) measurements, which confirmed a cell viability > 90% in each sample. Data were collected using the FACSDiva™ software (BD Biosciences) and analyses were performed using the Kaluza software (Beckman Coulter). The results of L31 and DT9 mAbs were expressed as Relative Median Fluorescence Intensity (RMFI) calculated as reported below, using the MFI (Median Fluorescence Intensity) values:

$$RMFI = \frac{MFI\ sample - MFI\ control}{MFI\ control}$$

The fold change was calculated as ratio between RMFI after and prior to acid wash for the L31 mAb, and prior to and after acid wash for the DT9 mAb.

PBMC western blotting analyses

PBMC proteins were extracted using the RIPA Buffer and were quantified at spectrophotometer (Eppendorf) using Coomassie Plus Bradford Protein Assay Reagent (ThermoScientific). To evaluate the expression of HLA-C 6 μg of PBMC proteins were treated with 3% β-mercaptoethanol and separated by SDS-PAGE on a 10% acrylamide gel. After proteins transfer on a PVDF membrane, western blot analyses were performed using L31 and anti α/β tubulin antibodies, diluted respectively 1:400 and 1:2000 in 5% milk TBS-Tween 0.05%. Anti-mouse IgG HRP (Promega) or anti-rabbit Ig goat conjugated HRP (Perkin Elmer) were used as secondary antibodies, both diluted 1:2000 in 5% milk TBS-Tween 0.05%. 6 μg of HEK-293T and CHO protein extracts were loaded in each gel. The first was used as internal standard and the second one as negative control. To evaluate the expression of β_2m were used 4 μg of PBMC protein extracts. Prior to the separation by SDS-PAGE on a 15% acrylamide gel, proteins were treated with 3% β-mercaptoethanol. After the transfer on PVDF membrane, western blot analyses were performed using anti β_2m and anti α/β tubulin antibodies, diluted respectively 1:400 and 1:2000 in 5% milk TBS-Tween 0.05%. Anti-rabbit Ig goat conjugated HRP (Perkin Elmer) was used as secondary antibody. 12 μg of HEK-293T β_2m positive and negative proteins extracts were loaded in each gel. The first was used as internal standard and the second one as negative control. The signal was developed using the ECL Advance™ Western Blotting Detection Kit (Amersham), through the AutoChemi System UVP (BioImaging System). The amount of HLA-C and β_2m were quantified through densitometric analysis using ImageJ Software. The values of each sample were normalized on their α/β tubulin values, and on the value of HEK-293T proteins. The ratio between HLA-C intensity and tubulin intensity both for the internal control (HEK-293T cells) and for each sample was calculated as:

$$Intensity\ (HLA-C)\ 293T\ normalized = \frac{Intensity\ (HLA-C)\ 293T}{Intensity\ (tubulin)\ 293T}$$

$$Intensity\ (HLA-C)\ sample\ normalized = \frac{Intensity\ (HLA-C)\ sample}{Intensity\ (tubulin)\ sample}$$

The ratio between these two values was used to establish the HLA-C expression level of each sample.

$$HLA-C\ sample\ value = \frac{Intensity\ (HLA-C)\ sample\ normalized}{Intensity\ (HLA-C)\ 293T\ normalized}$$

The same formula was applied for the β_2m quantification.

Infection of PBMC and TZM-bl cells

PBMC infections were carried out in the BSL 3 Human Virology Laboratory directed by Mauro Malnati (San Raffaele Hospital, Division of Immunology, Transplantation and Infectious Diseases, Milan, Italy) with the collaboration of Dr. Priscilla Biswas and Dr. Francesca Sironi. Viability of all thawed cells was checked by trypan blue dye exclusion and was routinely about 95-98%. PBMC were activated with phytoemagglutinin (PHA) (5 µg/ml) for 48 hours. Cells were infected with titred preparations of HIV-1 BaL (R5 or CCR5-user) and IIIB (X4 or CXCR4-user) (MOI range: 0.5-3) and incubated at 37°C. Excess virus was washed away and PBMC were plated at 10^5/well and maintained in complete RPMI medium plus IL-2 (Proleukin, Novartis) at 200 U/ml. PBMC culture supernatants were harvested at day 8-12 post-infection and frozen at -20°C until the p24 quantification. Supernatants from HIV-infected PBMC were normalized for p24 content, and different input concentrations were used to infect TZM-bl target cells. Cultures were performed in triplicate and infection was assessed after two days of culture.

HLA-C*06 and -C*07 expressing 721.221-CD4 cells preparation

HLA-C*06 and HLA-C*07 sequences were obtained by retro-transcription of RNA extracted from HLA-C homozygous cell lines, MGAR and LBF, respectively (kindly provided by Patrizio Giacomini, Regina Elena Hospital, Rome, Italy). After cloning into pcDNA 6.2 vector, sequences were transfected into 721.221-CD4 cells.

HLA-C*06 and -C*07 expressing 721.221-CD4 cells cytofluorimetric analyses

721.221-CD4 cells were transfected either with the HLA-C*06 or the HLA-C*07 encoding plasmid. Before cytofluorimetric analysis cells were treated with acid wash. Thus, cells were re-suspended in culture medium, and then washed with PBS. Cells were stained for 40 minutes at 4°C with 1 μg/ml L31 antibody. After washing with PBS, cells were labelled with APC conjugated goat anti-mouse antibody, diluted 1:200 in 5% w/v BSA in PBS, for 30 minutes at 4°C. After PBS wash, cells were analysed with FACSCantoTM flow cytometer and data were collected using the FACSDivaTM software (BD Biosciences). The fluorescence fold change ratio was calculated as the ratio between RMFI after and prior to acid wash.

Infection of 721.221-CD4 and TZM-bl cells

721.221-CD4 infection was performed in the BSL 3 Human Virology Laboratory (San Raffaele Hospital, Division of Immunology, Transplantation and Infectious Diseases, Milan, Italy).

Both 721.221-CD4-C*06 and 721.221-CD4-C*07 were incubated overnight with titred preparations of the X4-tropic HIV-1 IIIB isolate (MOI range: 0.5-3) at 37°C. 721.221-CD4 culture supernatants were harvested at day 3 post-infection and frozen at -20°C until the p24 quantification. Four different input of p24

concentrations were used to infect target TZM-bl cells. Triplicate cultures were set up and the experiment was repeated twice.

Viral replication assay

The HIV-1 p24 Gag protein was measured by a twin-site sandwich enzyme-linked immunosorbent assay (ELISA) (Aalto Bio Reagents Ltd, Dublin, Ireland), based on a previously published method (Moore, McKeating et al. 1990). Briefly, p24 antigen was captured from a detergent lysate of virions present in culture supernatants by a sheep polyclonal antibody adsorbed to a solid phase (3 hours incubation at room temperature). Bound p24 was detected with a mouse alkaline phosphatase-conjugated anti-p24 mAb (1 hour incubation) and a luminescent detection system. The luminescence was measured by the Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany) yielding Relative Luminescence Units (RLU). Using an internal p24 standard curve, the RLU were converted to ng/ml values. TZM-bl cells were lysed with 0.5% NP-40 (15 minutes at 37°C), then 50 µl of lysate was transferred to a 96 well-flat bottom plate followed by addition of the β-Gal substrate chlorophenol red-β-D-galactopyranoside (CPRG) (Roche Applied Sciences) at 5 mg/ml. The absorbency was read at 570 nm with an ELISA microplate reader (Biorad 680) and values expressed as OD/min.

Time course analysis of HLA-C/β_2m/peptide dissociation rate

A3.01 cells were treated for different times with acid wash (RPMI-1640, 20% FBS, pH 2.5). After 1, 2, 3 and 4 minutes of treatment on ice, cells were rinsed in culture medium, and then washed with PBS. Cells were stained with 1 µg/ml of mAb L31 incubating for 40 minutes at 4°C. After PBS wash, cells were stained with the secondary antibody: the APC-conjugated anti-mouse, diluted 1:200 in 5% w/v BSA in PBS. Data were acquired using the FACSCantoTM flow

cytometer and analysed using Kaluza software (Beckman Coulter). The fluorescence fold change ratio was calculated as the ratio between RMFI after and prior to acid wash.

Production of Env-pseudotyped viruses and TZM-bl assay in the presence of β_2m

$1x10^6$ HEK-293T cells were transfected with 1.5 μg of the Env plasmid DNA and 3 μg of the pSG3$^{\Delta env}$ backbone plasmid. 48 hours after the transfection, the medium was collected, cellular debris were eliminated by centrifugation and FBS was added to reach 20% final concentration. Two different viral dilutions (1:250 and 1:500) were used to infect $1x10^4$ TZM-bl target cells in the presence of 15 μg/ml DEAE-dextran (Li, Gao et al. 2005) and 3-fold dilutions of soluble β_2m (from 3 to 0.01 μg/ml). After 48 hours from infection, luminescence was measured using the Victor^{TM} 3 luminometer (Perkin Elmer) and infectivity was expressed as RLU. The experiment was performed in quadruplicate.

Infectivity assay of Env-pseudotyped viruses produced in the presence of different antibodies

$1x10^6$ HEK-293T cells were transfected with 1.5 μg of the Env plasmid DNA and 3 μg of the pSG3$^{\Delta env}$ backbone plasmid. After 6 hours from the transfection an anti β_2m (BBM1) or an unrelated anti PTPRG (PTPRG) antibody was added. Both antibodies were used concentrated 3 μg/ml.
48 hours after the transfection, the medium was collected, cellular debris were eliminated by centrifugation and FBS was added to reach 20% final concentration. 5-fold viral dilutions were used to infect $1x10^4$ TZM-bl target cells in the presence of 15 μg/ml DEAE-dextran (Li, Gao et al. 2005). After 48 hours from infection luminescence was measured using the Victor^{TM} 3 luminometer (Perkin Elmer) and infectivity was expressed as RLU. The experiment was performed in quadruplicate.

Statistical analyses

Statistical analyses were performed with the collaboration of Prof.ssa Lucia Cazzoletti (Department of Diagnostics and Public Health, University of Verona, Verona). Data were represented by percentages for categorical variables, and as means or median values for continuous variables, when appropriate. Two-way ANOVA was used to compare differences between β_2m positive and negative Env-pseudotyped virions, with the viral infectivity as dependent variable, the β_2m presence and the viral concentration as independent variables. Comparison of variables between subjects harboring HLA-C alleles belonging to the Stable/Unstable group was performed by χ^2 test for categorical variables and the t-test or the Wilcoxon test for continuous variables. Three-way ANOVA and Two-way ANOVA were used to ascertain significances between Stable and Unstable HLA-C alleles in determining viral infectivity. Experimental set and viral dilutions and experimental set only were respectively used as factor of variability. Two-way ANOVA was used to compare differences between HLA-C*07 and HLA-C*06 expressing cells in the controlled cellular model, with the fluorescence fold change ratio as dependent variable, the HLA-C allele and the experiment replicas as independent variables. Two-way ANOVA was also used to evaluate the difference in HIV-1 IIIB infectivity in 721.221-CD4 cells, with the viral infectivity as dependent variable, the HLA-C allele and the viral concentration as independent variables. Two-way ANOVA was used to compare the differences between HIV-1 QHO Env-pseudotyped viruses infectivity in the presence of different concentrations of exogenous β_2m. Three-way ANOVA was used to evaluate differences in pseudoviruses infectivity produced in the presence of different antibodies. The conventional 5% level of statistical significance was used. Data were analysed using StataMP 14.0 (Stata Corp., College Station, TX, USA).

Author contributions

All the experiments reported in the present thesis were performed by the Ph.D student Dr. Francesca Parolini except for the viral infections, which were conducted in the biosafety level 3 (BSL 3) Human Virology Laboratory, directed by Dr. Mauro Malnati, at San Raffaele Hospital, with the collaboration of Dr. Francesca Sironi and Dr. Priscilla Biswas. The experiments reported in the paper entitled "HIV-1 Env associates with HLA-C free-chains at the cell membrane modulating viral infectivity" (Serena et al, Scientific Reports 2016) were conducted with the Dr. Michela Serena, Ph.D fellow.

Donor samples were collected at the UOC Transfusional Medicine, AOUI Verona, directed by Dr. Giorgio Gandini, with the collaboration of Dr. Elisabetta Guizzardi and Dr. Valentina Muraro.

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Abstracts presented during PhD cycle XXX

IN SILICO STRUCTURAL PREDICTION AND IN VITRO FUNCTIONAL VALIDATION IDENTIFY THE ACOT8 REGIONS INVOLVED IN THE INTERACTION WITH HIV-1 NEF

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Introduction

HIV-1 Nef interacts with several cellular proteins, among which the human peroxisomal thioesterase 8 (ACOT8). This interaction appears to influence the CD4 down-regulation and might modulate lipid composition of membrane proteins during HIV-1 infection.

The Nef regions involved in the association with ACOT8 have been experimentally characterized. The lack of structural information for ACOT8 limits the full comprehension of the biological role of the Nef/ACOT8 association relevant to HIV-1 infection.

Results

In this work we modelled, through *in silico* predictions, the ACOT8 structure. A high charge complementarity was observed between Nef and ACOT8 surfaces. This allowed the identification of the ACOT8 aminoacids most likely involved in the interaction with Nef. They map in the Arg^{45}-Phe^{55} and Arg^{86}-Pro^{93} ACOT8 regions. Their role has been validated by *in vitro* assays through the development of ACOT8 deletion mutants.

Immunofluorescence and co-immunoprecipitation analyses showed that the ACOT8 K91S mutation is sufficient to abrogate the interaction with Nef. In addition, the ACOT8 Arg^{45}-Phe^{55} region, as well as the Arg^{86}-Pro^{93} region, are involved in Nef binding.

Conclusions

Our data demonstrate that the ACOT8 Lys^{91} plays a key role in the interaction with Nef. The observation that both ACOT8 Arg^{45}-Phe^{55} and Arg^{86}-Pro^{93} regions are determinant for Nef association suggests that the interaction involves a wider region on ACOT8 surface. These findings improve the comprehension of the association between HIV-1 Nef and ACOT8 and will help elucidating the biological meaning of their interaction.

Graduate School for Health and Life Sciences

PhD DAY
Friday 29th January, 2016

CRISPR/CAS9: A NEW TOOL FOR GENOME EDITING

Development of β_2m knock out cell lines

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Introduction

The CRISPR/Cas9 system is a new, promising technique that allows editing of DNA sequences in the cell genome. The Cas9 endonuclease is guided to specific target locations within complex genomes by a short RNA search string (gRNA) (Patrick D. Hsu et al, Cell, 157, 1262, 2014). In this work I used the CRISPR/Cas9 system to originate different β_2-microglobulin (β2m) knock out cell lines to elucidate the role of β2m and HLA-C in HIV-1 infection.

Methods

Hek-293T, HeLa and HeLa-Lai cells were cotransfected with a plasmid for the β_2m gRNA (Pankaj K. Mandal et al, Cell Press, 15, 643, 2014) and the PX459 V2.0 plasmid (Addgene) to obtain β_2m knock out cell lines. Following puromycin selection and expansion, cells were negatively sorted in two rounds, first with the anti-β_2m antibody NAMB-1 and then with the anti-MHC-I antibody W6/32. The absence of β_2m expression in sorted cells was confirmed by immunofluorescence and Western blot. Different HIV-1 pseudoviruses were produced following Montefiori's Protocol (Ming Li et al, J Virol, 79, 10108, 2005).

Results

Pseudoviruses produced in Hek-293T β_2m negative cells show a significant lower infectivity than the corresponding pseudoviruses produced in β_2m expressing cells. HIV-1 pseudoviruses used were QHO and pRHPA, while VSV-G pseudovirus was used as negative control.

Flow cytometry on β_2m-negative HeLa and HeLa-Lai cells show the absence of HLA-C expression on the cell membrane, indicating that the presence of HIV-1 Env (in HeLa-Lai cells) does not have any effect on the HLA-C translocation pathway, which strictly requires β_2m as a chaperon.

Conclusions

The CRISPR/Cas9 system is a powerful tool for specific genome editing to originate knock-out mutants in different cell lines.

Obtained results demonstrate that 1) the absence of β_2m causes the absence of HLA-C on HIV-1 virions; β2m and HLA-C defective virions are significantly less infectious (about 5 times); 2) HIV-1 Env can't restore HLA-C expression on the cell surface in the absence of β_2m. Most likely, HLA-C can bind HIV-1 Env only when it is detached from β_2m on the cell surface.

These findings improve the comprehension of the role of HLA-C in modulating HIV-1 infectivity.

CRISPR/CAS9 AS A TOOL FOR STUDYING THE INTERACTIONS BETWEEN VIRAL AND CELLULAR PROTEINS

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The CRISPR/Cas9 system is a new, promising technique that allows editing of DNA sequences in the cell genome.

We used CRISPR/Cas9 to produce $\beta_2$microglobulin (β_2m) and human thioesterase 8 (ACOT8) knock-out cell lines to study their role in HIV-1 infection. HLA-C is translocated at the cell surface only when complexed with β_2m, where it interacts with HIV-1 Env resulting in increased viral infectivity (Zipeto & Beretta, Retrovirology 2012). ACOT8 is a human thioesterase interacting with HIV-1 Nef increasing Nef stability (Serena et al, Scientific Reports 2016).

The loss of β_2m expression in 293T, HeLa-Lai (expressing HIV-1 Env) and parental HeLa cells was assessed by western blot and flow cytometry and β_2m negative cells were sorted. Cytofluorimetric analysis indicated that, in the absence of β_2m, HLA-C expression on the cell membrane was abrogated, both in HeLa and in HeLa-Lai cells. The presence of HIV-1 Env did not restore HLA-C on the cell surface. This finding suggests that HLA-C needs β_2m to be expressed on the cell membrane, where the Env/HLA-C interaction occurs. In addition, we observed that HIV-1 viruses produced in 293T β_2m negative cells were three fold less infectious than those produced in parental cells, suggesting that the presence of HLA-C on the cell surface is required for the association with Env, which results in an increased virus infectivity.

The loss of ACOT8 expression in 293T and in TZM-bl cells was assessed by western blot. Cells were clonally expanded and ACOT8 knock out was further confirmed.

The role of Nef/ACOT8 association will be explored comparing the infectivity of HIV-1 viruses produced in the presence or in the absence of ACOT8 (293T), as well as comparing the infection of HIV-1 viruses in ACOT8 positive or negative host cells (TZM-bl).


AIBT
ASSOCIAZIONE ITALIANA DI IMMUNOGENETICA E BIOLOGIA DEI TRAPIANTI
XXIII Congresso Nazionale
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nel futuro dei trapianti
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Aula Magna del Dipartimento di Scienze Umane dell'Università degli Studi dell'Aquila

IL RUOLO DI HLA-C NELL'INFEZIONE DA HIV-1

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Introduzione: I livelli di espressione cellulare degli alleli HL A -C sono correlati al controllo dell'infezione da HIV-1; molecole HLA-C codificate da alleli considerati protettivi (HLA-C*02, C*05, C*06, C*08, C*12, C*15, C*16), potrebbero controllare meglio l'infezione. Al contrario, molecole HLA-C codificate da alleli considerati non protettivi (HLA-C*01, C*03, C*04, C*07, C*14), potrebbero favorire la rapida progressione verso l'AIDS. La principale caratteristica delle molecole HLA-C risiede nella loro scarsa efficienza di assemblaggio, che risulta in un accumulo come *Free Chains*. Dati preliminari suggeriscono che la presenza di molecole HLA-C *Free Chains* possa contribuire a modulare l'infettività dei vironi di HIV-1, in seguito ad associazione con la proteina Env dell'*envelope* virale. Il ruolo protettivo che deriva da un elevato livello di espressione di HLA-C contrasta con l'aumentata infettività di HIV-1 quando HLA-C viene incorporata nel virione. Questa contraddizione potrebbe essere il risultato della presenza di diverse conformazioni di HLA-C; la molecola, infatti, potrebbe associarsi in maniera mutualmente esclusiva o con la β_2m oppure con la proteina Env di HIV-1.

Pazienti e metodi: Sono stati esaminati i risultati della genotipizzazione di donatori periodici di sangue iscritti al Registro IBMDR nel triennio 2013-2015 presso l'UOC di Medicina Trasfusionale dell'AOUI Verona. I donatori sono stati selezionati al fine di escludere alleli HLA-B* reattivi con gli anticorpi utilizzati e di includere soggetti con entrambi i loci HLA-C* appartenenti al gruppo di alleli definiti protettivi o non protettivi. Scopo del lavoro è stato analizzare il rapporto esistente fra molecole HLA-C associate alla β_2m e molecole HLA-C *Free Chains* su PBMC dei donatori. A tal fine è stata verificata l'espressione in membrana del complesso completo formato da HLA-C/β_2m/peptide e di HLA-C come F*ree Chain*. È stata, inoltre, esaminata la quantità totale di molecole HLA-C prodotte dai vari soggetti e, per verificare la stabilità di legame tra HLA-C e β_2m, è stato analizzato il *Fold change* di fluorescenza. Infine, per investigare l'infettività di HIV-1 in presenza di differenti alleli HLA-C sono stati condotti esperimenti di infezione di PBMC *in vitro*.

Conclusioni: Soggetti con alleli HLA-C non protettivi/instabili sono caratterizzati dalla presenza di maggiori quantità di HLA-C *Free Chains*, necessarie al virus per essere più infettivo. Al contrario, soggetti con alleli HLA-C protettivi/stabili presentano un maggior numero di complessi completi in membrana in grado di stimolare l'immunità cellulare e, quindi, controllare meglio l'infezione da HIV-1. Questi risultati forniscono un'ulteriore evidenza riguardo al ruolo di diverse varianti genetiche implicate nel controllo dell'infezione da HIV-1. La caratterizzazione delle varianti alleliche HLA-C potrebbe contribuire al miglioramento delle terapie antivirali, tenendo conto della variabilità genetica individuale.

St George's, University of London Annual Research Day

Wednesday 30 November 2016

Morning lecture session: Michael Heron Lecture Theatre

Programme: 'Genomics and Human Health'

8.15 to 9.15	**Arrival and Registration – *Meeting rooms 3&4, 2nd floor Hunter Wing***
9.20	Welcome and Introduction – Michael Heron Lecture Theatre Professor Jenny Higham, Principal, St George's, University of London
9.30	**Professor Sahar Mansour (St George's University Hospitals NHS Foundation Trust)** 'Genomics of rare diseases- new genes for lymphovascular disorders'
10.10	**Professor David Strachan (St George's, University of London)** 'Genes and environment in asthma and allergy: GWAS, GWIS and 'Gee! Was that it?'
10.50	Break
11.20	**Chair: Professor Jodi Lindsay, St George's, University of London** **Professor Nick Thomson (Sanger Institute, Cambridge and London School of Hygiene and Tropical Medicine)** 'From global to local: using phylogenomics to follow bacterial infection and spread'
12.00 12.10	**The Thomas Young Prize Lecture** **Chair: Professor Mark Fisher, Dean of Research, St George's, University of London** **Professor Hagan Bayley FRS (University of Oxford)** 'Engineered nanopores for sensing and sequencing of DNA, proteins and sugars'
13.00	Lunch (Alistair Hunter Room)
14.00	Poster session and trade exhibition Venue: Boardrooms
16.00	Presentations: Chrissie Fenske Research Poster Prize and other awards Venue: Boardrooms
17.00	Close of meeting

HLA-C EXPRESSION LEVELS AND BINDING STABILITY TO β_2m INFLUENCE HIV-1 INFECTIVITY

Parolini F.[1], Biswas P.[2], Serena M.[1], Sironi F.[2], Muraro V.[3], Guizzardi E.[3], Cazzoletti L[4]., Cavallini C.[5], Scupoli MT.[5], Malnati M.[2], Beretta A.[2], Romanelli MG.[1], Zipeto D[1]

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Different HLA-C expression levels lead to different HIV-1 infection outcomes: a higher expression leads to an effective infection control. But the absence of HLA-C on the envelope causes a decrease in the infectivity. HLA-C alleles show different binding stability to β_2m: the highly expressed are more stably bound to β_2m than the lowly expressed. Since HIV-1 interacts with HLA-C free chains we resolved this contradiction focusing on the HLA-C/β_2m binding stability. Thus we divided HLA-C alleles in 2 groups: High/Stable, Low/Unstable. We analyzed the stability by calculating the ratio between the reactivity to specific antibodies of HLA-C bound or not to β_2m on PBMC. This ratio was analyzed with the L31 antibody, specific for HLA-C free chains, before and after an acid wash, to detach the β_2m from HLA-C. Its value is higher in High/Stable group, confirming that these alleles bind tightly to β_2m. Finally HIV-1 produced in Low/Unstable PBMC is more infectious. We propose that the HLA-C expression level and its β_2m binding stability play a role in the HIV-1 outcome. High/Stable alleles are associated with better immune control and lower HIV-1 infectivity, while Low/Unstable ones with worse immune control and higher infectivity.


Graduate School for Health and Life Sciences
PhD DAY
Thursday 15th December, 2016
Program
9.00 – 10.30
Poster attendance: 1st 2nd and 3rd year PhD students
Ground floor and room-1 – Lente Didattica Policlinico GB Rossi
10.30 – 10.45
Tea / coffee break
10.45 – 12.45
Guided poster sessions
12.45 – 13.45
Buffet offered by the Graduate School
13.45 – 14.45
Introductory remarks and Invited Lecture
Aula 1 "Roberto Vecchioni" – Lente Didattica – Policlinico G.B Rossi
Prof. Antonio Lupo – Vice-Rector
Prof. Alfredo Guglielmi – Director of the School of Medicine
Prof. Michele Muggeo – Professor Emeritus, Università di Verona
Prof. Carlo Patrono Professor of Pharmacology
Università Cattolica del Sacro Cuore di Roma
"Low-Dose Aspirin, Coronary Atherothrombosis, and Colorectal Cancer"
14.45 – 16:45
Oral presentations: 2nd and 3rd year PhD students
TEDMED talks:
Dott. Naseer AHMED PhD Program in Cardiovascular Sciences
Dott.ssa Elisabetta BENEDUCE PhD Program in Biomolecular Medicine
Dott.ssa Dalila BEVILACQUA PhD Program in Inflammation, Immunity and Cancer
Dott.ssa Nicole CORSI PhD Program in Neuroscience, Psychological and Psychiatric Science
Dott. Alessandro GATTI PhD Program in Clinical and Experimental Biomedical Sciences
Dott.ssa Angela SANDRI PhD Program in Applied Life and Health Sciences
Closing remarks and awards distribution
Prof. Mario Pezzotti – Deputy Rector for Scientific Research
Prof. Pietro Minuz – Director of the Graduate School
Poster placement : 8.30 – 9.00 (Room 3 e 4 – Lente Didattica)

HLA-C EXPRESSION LEVELS AND BINDING STABILITY TO β_2m INFLUENCE HIV-1 INFECTIVITY

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Introduction:

Different HLA-C expression levels lead to different HIV-1 infection outcomes: a higher expression leads to a better antigen presentation to CTLs and thus to an effective HIV-1 infection control. But HIV-1 needs the presence HLA-C on the viral envelope to increase its infectivity. Furthermore, HLA-C alleles show different binding stability to β_2m: the highly expressed ones are more stably bound to β_2m than the lowly expressed ones.

Experiments and results:

HIV-1 reactivation increases the amount of HLA-C free chains not bound to β_2m in PM1 (a HIV-1 III-B chronically infected human T-lymphocytic cell line) and in ACH2 cells (A3.01 cells harboring HIV-1 genome). We transfected 293T cells with an HIV-1 Env plasmid and an Env defective full length HIV-1 genome plasmid, pSG3Δenv. An HLA-C free chains increase was observed after Env transfection, whereas no differences in the total amount of HLA-C on the cells surface were observed . This suggest that Env induces a conformational switch in HLA-C. We found that HIV-1 viruses generated in 293T β_2m negative cells, obtained using CRISPR/Cas9 system, are less infectious than those produced in the presence of β_2m. Since the protective role of highly expressed alleles appears to be in contrast with these data suggesting an Env/HLA-C role in increasing HIV-1 infectivity, we analysed HLA-C stability. We divided HLA-C alleles in 2 groups: High/Stable and Low/Unstable. We analysed this stability by calculating the ratio (fluorescence fold change) between the L31 (a mAb recognizing HLA-C free chains) reactivity before and after acid wash on PBMC donors to remove β_2m. A higher fold change was observed when analyzing high/stable HLA-C alleles, confirming that these alleles bind more tightly to β_2m. We finally showed that HIV-1 virions produced in PBMC with Low/Unstable alleles are more infectious.

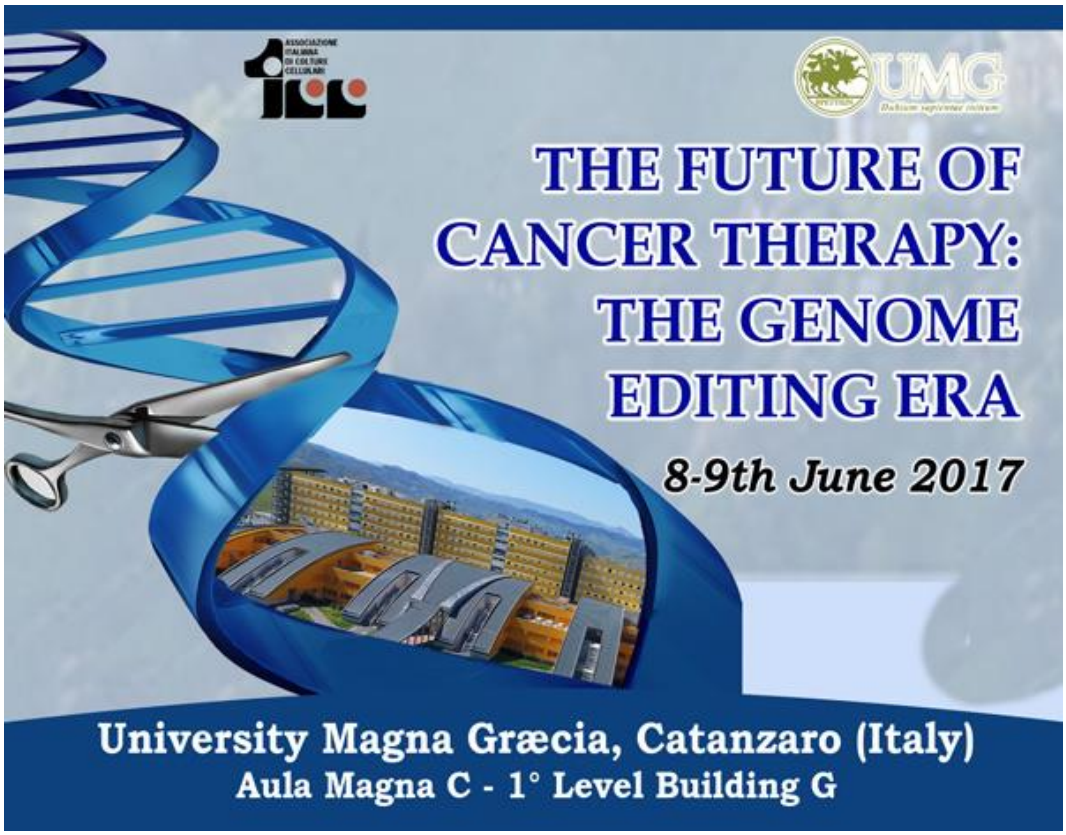
Conclusions:

We propose that HLA-C expression level and its β_2m binding stability play a key role in HIV-1 infection control. High/Stable alleles are associated with better immune control and lower viral infectivity, while Low/Unstable ones with worse immune control and higher infectivity.

CRISPR/CAS9 AS A POWERFUL TOOL FOR GENOME EDITING

F. Parolini, S. Mutascio, M. Serena, S. Fochi, M.G. Romanelli, D. Zipeto
Department of Neurosciences, Biomedicine and Movement Sciences University of Verona, Italy

CRISPR/Cas9 is a prokaryotic molecular immunity system that exploits short RNAs to degrade complementary DNA sequences of invading bacteriophages (Church, 2013). This system has been deeply characterized and engineered to be exploited for genome editing in eukaryotic cells. CRISPR/Cas9 introduces specific double strand breaks in DNA molecules, activating two possible cellular repair responses. In the absence of a donor template, Non-Homologous End Joining repair is activated, leading to insertion/deletion mutations that inactivate the target gene. In the presence of a homologous donor template, the damage is repaired triggering the Homologous Recombination pathway that inserts the donor sequence in the target locus (Hsu, 2014). The CRISPR/Cas9 system has been applied, among others, to study and counteract viral infections. CRISPR/Cas9 was exploited by Van Diemen (2016) to target the EBNA1 gene, reducing EBV latency. Similar results were obtained on HBV (Ramanan, 2015) and on HSV-1, by targeting the immediate-early genes, abrogating HSV-1 infectivity (Roehm, 2015). To avoid concerns associated with the use of porcine organs for transplantations, Yang (2015) used CRISPR/Cas9 to remove endogenous retroviruses from porcine cells. CRISPR/Cas9 was used to inactivate HPV-16/18, responsible of cervical carcinoma, by targeting the E6 and E7 genes (Kennedy, 2014; Zhen, 2014). The E7 gene was inactivated in HPV-6/11, the main causes of genital warts, within transformed keratinocytes (Liu, 2016).Several efforts were employed to contrast HIV-1 infection. Since the TAR sequence is conserved among different virus subtypes, it was chosen as a target for CRISPR/Cas9 (Liao, 2014; Kaminiski, 2016). Zhang (2015) targeted the RNA polymerase promoter within the 5' LTR region, while Zhu (2015) inactivated the HIV-1 regulatory Rev gene. Noteworthy, to avoid the generation of HIV-1 escape mutants (Liang, 2016), a different strategy, targeting the CCR5 co-receptor, was employed (Kang, 2015). Our research group is studying the interactions between HIV-1 and cell host proteins. To define the role of MHC-I molecules in modulating HIV-1 infectivity, we developed $\beta_2$microglobulin knock out cell lines. HIV-1 virions produced in $\beta_2$microglobulin negatives cells were found to be less infectious (Serena, 2017). In addition, we showed that the HIV-1 Nef protein is stabilized by the human peroxisomal thioesterase 8 (ACOT8). To investigate the role of this interaction we developed ACOT8 negative cells to be used either for virus production and for viral infection. CRISPR/Cas9 is thus a powerful system to study the interactions between viruses and host, as well as a promising therapeutic tool to fight viral infections.


THE FUTURE OF CANCER THERAPY: THE GENOME EDITING ERA
8-9th June 2017
University Magna Græcia, Catanzaro (Italy)
Aula Magna C - 1° Level Building G

CRISPR/CAS9 FOR THE STUDY OF THE INTERACTIONS BETWEEN VIRUSES AND HOST

Parolini F., Mutascio S., Serena M., Fochi S., Romanelli M. G., Zipeto D.
Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona

The CRISPR/Cas9 system has many applications in virology: it has been used to achieve viral DNA inactivation from latently infected cells, allowing viral eradication, or to inactivate specific proteins involved in virus-host cell interaction. Herein we applied the CRISPR/Cas9 technique to generate knock-out cell lines useful for the study of cellular determinants critical for HIV-1 infection. As a preliminary screening, the editing efficiency was evaluated by T7 endonuclease I assay, and then confirmed by western blot and flow cytometry analyses. We targeted $\beta_2$microglobulin (β_2m), human thioesterase 8 (ACOT8) and histone deacetylase 6 (HDAC6) genes. $\beta_2$microglobulin is required for the membrane translocation of HLA molecules where HLA-C interacts with HIV-1 Env and modulates viral infectivity (Zipeto & Beretta, Retrovirology 2012). We edited β_2m in 293T, HeLa-Lai (expressing HIV-1 Env), TZM-bl (CD4 and CCR5 expressing HeLa, highly sensitive to HIV-1 infection) and parental HeLa cells. We showed in 293T cells that HIV-1 proteins transfection did not translocate HLA-C at the cell surface in absence of β_2m. We obtained similar result in β_2m negative HeLa-Lai cells, showing that HIV-1 Env interacts with HLA-C at the plasma membrane after its surface translocation. Besides, we demonstrated that HIV-1 pseudoviruses produced in β_2m negative 293T cells were significantly less infectious than those produced in parental ones (Serena et al., Scientific Reports, 2017). ACOT8 thioesterase interacts with HIV-1 Nef protein preventing its degradation (Serena et al, Scientific Reports 2016). To better understand the role of ACOT8 in HIV-1 infectivity, we developed ACOT8 knock out 293T and TZM-bl cell lines. We observed in TZM-bl cells, susceptible to HIV-1 infection, that ACOT8 absence did not affect the infectivity. The role of ACOT8 in pseudoviruses production is being tested using 293T edited cells. HDAC6 is an important regulator of membrane dynamics involved in HIV-1 infection (Valenzuela-Fernandez et al, Molecular biology of the cell, 2005). We inactivated the HDAC6 gene in 293T cells. These cells will be used to test the HIV-1 infectivity and syncytia formation. In conclusion, the CRISPR/Cas9 system represents a new, powerful tool in basic and applied research in virology.

A CRISPR/CAS9 BASED APPROACH TO STUDY THE IMPLICATION OF HTLV REGULATORY PROTEINS IN THE NF-κB MODULATION

Stefania Fochi, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli
Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona

Human T-cell leukemia virus type 1 (HTLV-1) infects approximately 20 million people worldwide and 5% of them may develop adult T-cell leukemia (ATL), a fatal T-cell malignancy with no effective treatment currently available. The homologous HTLV-2 does not cause ATL, but is associated with milder neurologic disorders. Both viruses encode a potent viral oncoprotein, termed Tax, which deregulates several cellular pathways, including NF-κB. In addition to Tax, the HTLV-1 proviral genome encodes from the antisense strand, a basic leucin zipper factor, HBZ, which plays an essential role in the oncogenic process leading to ATL. Comparative studies of the functional activity of Tax-1 and HBZ, and the HTLV-2 homologous, Tax-2 and APH-2 (HTLV-2 antisense protein), may provide clues to explain the dissimilar pathobiology of HTLVs. Herein, we compared the effect of the viral regulatory proteins HBZ and APH-2 on Tax-modulated NF-κB cell signaling. Our data demonstrated that APH-2 suppressed, more efficiently than HBZ, the Tax-dependent NF-κB activation. By confocal microscopy, we observed that, differently from HBZ, the APH-2 protein is recruited into cytoplasmic structures where co-localized with Tax. The co-expression of APH-2 and Tax impaired the degradation of the NF-κB inhibitor IκB-α, restraining the transcriptional factor p65 into the cytoplasm. APH-2, but not HBZ, was present in complex containing the TRAF3 protein, an upstream inhibitor of the alternative NF-κB pathway. Applying the CRISPR/Cas9 technique, we generated TRAF3 knock-out cell lines. Several $TRAF3^{-/-}$ clones were selected and NF-κB promoter activity was analyzed by luciferase assays. The results showed that, in absence of induction, the NF-κB promoter is slightly activated, in the $TRAF3^{-/-}$ cell line compared to the parental cell line. The absence of TRAF3 adaptor factor did not inhibit the Tax-mediated NF-κB activation. Ongoing studies using $TRAF3^{-/-}$ clones will allow to clarify the effect of the HTLV antisense protein on the alternative NF-κB pathway activation.

CRISPR/CAS9 MEDIATED KNOCK-OUT OF RUNX2 IN MELANOMA CELLS

Valenti, Mt[1]; Zipeto, D[2]; Deiana, M[1]; Serena, M[2]; Parolini, F[2]; Cheri, S[1]; Gandini, A[3]; Mina, M[4]; Mutascio, S[2]; Viser, A[1]; Dalle Carbonare, L[1]

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Incidence of MM has increased considerably in consequence of lifestyle and environmental changes. The mortality rate for MM is very high as it is highly invasive and also genetically resistant to chemotherapeutic treatments. It has been reported that mutation rate and gene modulation in melanoma are higher than in other solid malignancies. In addition, transcription factors by acting on gene expression can affect cellular process. In particular a higher expression of RUNX2 in melanoma than in normal melanocytes have been shown. RUNX2 is overexpressed in several tumor tissues, including pancreatic cancer , breast cancer, ovarian epithelial cancer, prostate cancer , lung cancer and osteosarcoma. As no direct RUNX2 inhibitor is available and experiments performed with RNA interference were scarcely reproducible we applied Crisp/cas 9 technology to knockout the RUNT domain of RUNX2 in melanoma cell line. Crisp/Cas 9 tecnology was able to delete, partially, the RUNT domain. The deleted clone showed a reduced proliferation, reduced EMT features, reduced migration ability, suggesting the involvement of RUNT in different pathway of MM. In addition, the deleted clone showed a reduction of genes involved in migration ability and an increased expression of SSBP1 gene suggesting RUNT as oncotarget in MM.

The Institute of Genetic and Biomedical Research of CNR in collaboration with the Technology Park of Sardinia, the European School of Genetic Medicine and the University Residential Centre of Bertinoro organize the

The Punic-Roman remains of the ancient town of Nora next to the School venue

CRISPR/CAS9 GENOME EDITING IN MELANOMA CELLS: NEW INSIGHT OF RUNT DOMAIN

Cheri, S[1]; Zipeto, D[2]; Deiana, M[1]; Serena, M[2]; Parolini, F[2]; Gandini, A[3]; Mina, M[4]; Mutascio, S[2]; Visser, A[1]; Mottes, M[2]; Dalle Carbonare, L[1]; Valenti, MT[1]

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[4] Department of Medicine, Medical Oncology Section, University of Verona

Incidence of melanoma has increased considerably in Western population in consequence of lifestyle and environmental changes. The mortality rate is very high as it is highly invasive and also genetically resistant to chemotherapeutic treatments. It has been reported that mutation rate and gene modulation in melanoma are higher than in other solid malignancies. In addition, transcription factors by acting on gene expression can affect cellular process. In particular, a higher expression of RUNX2 in melanoma than in normal melanocytes have been shown. RUNX2 is the master gene of the osteogenic commitment of MSC, and it is overexpressed in several tumor tissues, including pancreatic cancer, breast cancer, ovarian epithelial cancer, prostate cancer, lung cancer and osteosarcoma. As no direct RUNX2 inhibitor is available and experiments performed with RNA interference were scarcely reproducible, we applied CRISPR/Cas9 technology, that avoid several of the pitfalls associated with interfering RNA, to knockout the RUNT domain of RUNX2 in melanoma cell line. CRISPR/Cas9 technology could delete, partially, the RUNT domain. The deleted clone showed a reduced proliferation, epithelial-mesenchymal transition features and migration ability, suggesting the involvement of RUNT in different pathways of melanoma. In addition, the deleted clone showed a reduction of genes involved in migration ability and an increased expression of SSBP1 gene proposing RUNT as an oncotarget in melanoma. In addition, the deleted clone showed a reduction of genes involved in migration ability and an increased expression of SSBP1 gene proposing RUNT as an oncotarget in melanoma.

CRISPR/CAS9 TO STUDY VIRUS-HOST INTERACTIONS

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Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Italy

The new CRISPR/Cas9 technique enables the editing of specific DNA sequences of any given genome. It has found many applications in virology, allowing, among others, the viral DNA excision from latently infected cells and the generation of useful knock out cell lines. Using the CRISPR/Cas9 system we originated $\beta_2$microglobulin (β_2m), human thioesterase 8 (ACOT8) and histone deacetylase 6 (HDAC6) negative cells, to study their role in HIV-1 infection. We evaluated the editing efficiency by the T7 endonuclease I assay, western blot and flow cytometry analyses. The β_2m is crucial for the HLA molecules membrane translocation. HLA-C interacts with HIV-1 Env on the cell membrane increasing viral infectivity (Zipeto & Beretta, Retrovirology 2012). We targeted the β_2m gene in 293T, HeLa-Lai (expressing HIV-1 Env), TZM-bl (HeLa cells susceptible to HIV-1 infection) and parental HeLa cells. We showed in 293T cells that β_2m absence abrogates HLA-C surface expression, even in the presence of HIV-1 proteins expression. We confirmed the observation in β_2m negative HeLa-Lai cells. Moreover, by comparing HIV-1 infectivity in β_2m positive/negative TZM-bl cells, we observed that virions produced in β_2m negative 293T cells are significantly less infectious than those produced in parental cells.

We reported that ACOT8 interacts with HIV-1 Nef preventing its degradation (Serena et al, Scientific Reports 2016). We observed in TZM-bl cells that ACOT8 absence did not affect HIV-1 infectivity. The ACOT8 role in HIV-1 production and infectivity is being tested using 293T ACOT8 negative cells.

HDAC6 is an important regulator of membrane dynamics involved in HIV-1 infection. We inactivated the HDAC6 gene in 293T cells to evaluate its influence on HIV-1 infectivity and syncytia formation and analyses are in progress.

In conclusion, the CRISPR/Cas9 genome edited cell lines are powerful tools to study the molecular interaction required for HIV efficient infection.

HOST-VIRUS INTERACTIONS: HTLV ANTISENSE REGULATORY PROTEINS PLAY A ROLE IN THE DYSREGULATION OF NF-KB PATHWAY

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Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL), an aggressive form of T-cell malignancy with no cure. The HTLV-1 oncoprotein Tax plays a key role in CD4+ T-cell transformation, mainly through constitutive activation of both the canonical and the alternative NF-κB pathways. The HTLV-1 basic zipper protein (HBZ), encoded by the antisense viral genome strand, plays an essential role in the oncogenic process in concert with Tax, mediating T-cell proliferation. Unlike HTLV-1, the genetically related retrovirus HTLV-2 is not associated with ATL diseases. Functional comparisons between HTLV-1 regulatory proteins, Tax-1 and HBZ, and the HTLV-2 homologs, Tax-2 and APH-2, may highlight different mechanisms of their oncogenic potential. The aim of this study is to investigate how the antisense proteins HBZ and APH-2 impaired the NF-κB pathway activation. We found that both HBZ and APH-2 antagonized the NF-κB promoter activity mediated by Tax, but not in the same extent. Analyzing the intracellular distribution of the antisense proteins, we found that APH-2 is retained in cytoplasm complexes, whereas HBZ is mainly distributed into the nucleus. We observed that in presence of APH-2 and Tax-2, the degradation of the IκB-α inhibitor was reduced. Moreover, we found that unlike HBZ, APH-2 formed complexes with an upstream inhibitor of the alternative NF-κB pathway, the TNF receptor-associated factor 3, TRAF3. We generated a TRAF3 knock-out cell line applying the CRISPR/Cas9-mediated genome editing. By luciferase assays, we showed that TRAF3 is not required for Tax mediated NF-κB promoter activation. Analyses are in progress to test the inhibitory effect of the antisense HBZ and APH-2 proteins on NF-κB promoter activity in absence of TRAF3. The results of this study may contribute to clarify the effect of the alternative NF-κB viral deregulation pathway in the expression of proinflammatory genes.

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HLA-C EXPRESSION LEVELS AND BINDING STABILITY TO β_2m MODULATE HIV-1 INFECTIVITY

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Background: HLA-C expression levels lead to different HIV-1 infection out- comes. A higher expression is associated with a better activation of cytotoxic T lymphocytes (CTLs) and thus a better HIV-1 infection control. Vice versa, a lower HLA-C expression leads to a rapid progression toward AIDS. Thus, HLA-C highly and lowly expressed alleles are defined as protective and non-protective, respectively. Furthermore, different HLA-C alleles have different binding stabilities to $\beta_2$microglobulin (β_2m). Interestingly, HLA-C protective alleles are also those that bind β_2m more efficiently, while the non-protective variants present more free chains (not bound to β_2m) on the cell surface. It is also known that virions lacking HLA-C have reduced infectivity and increased susceptibility to neutralizing anti- bodies.

Methods: The A3.01 cell line and its HIV-1-infected counterpart ACH-2 were used as an in vitro infection model. 293T β_2m negative cells, generated using CRISPR/ Cas9 system, were utilized to produce HIV-1 pseudoviruses. PBMC from healthy blood donors, harboring both protective or non-protective alleles, were exploited to characterize the proportion between HLA-C associated to β_2m and HLA-C presents as free chains on the cell surface. In addition, PBMC from the same donors were tested for their ability to support HIV-1 infection in vitro.

Results: HLA-C free chains, specifically more represented on the surface of infected cells, are responsible for the increase of virions' infectivity. We observed that HIV-1 Env-pseudotyped viruses produced in β_2m negative cells, thus lacking HLA-C on their envelope, are less infectious than those produced in the presence of β_2m. In PBMC we found that protective HLA-C variants are more stably bound to β_2m than non-protective ones and that HIV-1, in vitro, infects more efficiently PBMC harboring non-protective, weakly bound to β_2m, HLA-C alleles.

Conclusions: We propose that the outcome of HIV-1 infection might be driven both by the HLA-C surface expression levels and by the HLA-C/β_2m binding stability. According to this model, the expression of non-protective HLA-C alleles, which bind weakly β_2m, leads to a reduction of immunocompetent complexes expressed on the cell surface and to an increase of HLA-C free chains that raises viral infectivity, both leading to a rapid progression toward AIDS.

Publications

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